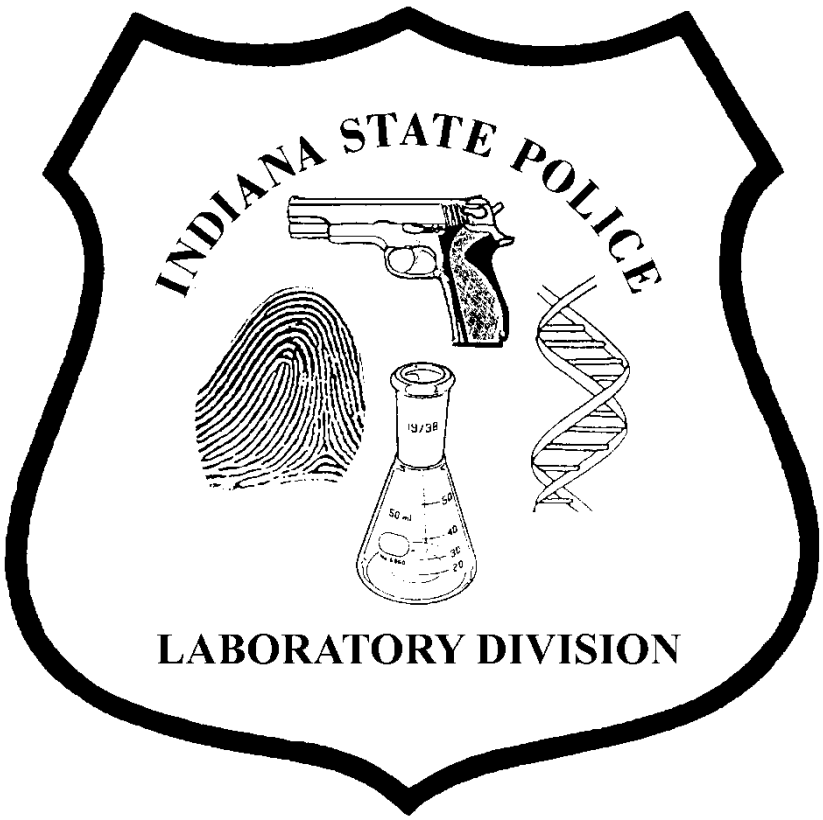
FORENSIC BIOLOGY SECTION



CASEWORK TEST METHODS

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

FORWARD

The Laboratory Division of the Indiana State Police (ISP) conducts tests on various body fluids, body fluid stains, and human hair for criminal justice agencies. DNA analysis is performed as needed on the various biological materials. The Laboratory reserves the right to evaluate and prioritize the items submitted and limit the total number in order to expedite service. The analysts of the Forensic Biology Section shall have a minimum of a baccalaureate or an advanced degree in a natural science or a closely related field. DNA analysts shall have successfully completed college course work covering the subject areas of genetics, biochemistry, molecular biology and statistics. All analysts undergo an intensive formalized training program dealing with forensic techniques and instrumentation. Completion of the Training Program is required before analysis of evidence is performed. Additionally, all analysts participate in proficiency testing utilizing open trials, blind trials, and/or re-examination techniques. The accuracy and specificity of test results are ensured by running known controls with each set of tests.

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1. Serology Methods:

1.1. Scope:

This test method is designed for the guidance of laboratory personnel who assist investigations where body fluids or stains occur as physical evidence in a variety of alleged criminal activities. The scope of this type of evidence includes but is not limited to the following: identification of blood, semen, animal species, and human hair. This test method may be expanded or altered as techniques and/or new genetic analyses are found applicable and validated.

1.1.1 Trace evidence including but not limited to hair, fibers, glass, or paint may be collected from evidence submitted for serological examination. Collected trace evidence shall be retained in the original packaging unless value is immediately apparent. Appropriate notes shall be taken on the trace evidence collected. When required, hairs shall be evaluated for the possibility of human origin and the potential for future analysis.

1.1.2 A search is made of submitted items for the presence of biological material. Once a questioned stain or material is detected and identified, if applicable, it is retained for possible DNA analysis.

1.1.3 Stain cards from whole blood samples, cuttings from body fluid stains and other DNA evidence shall be retained by the laboratory for any possible future testing. The retained items may be released at the prosecutor's request. The request shall be documented by the analyst in the case record.

1.1.4 A valid court order shall circumvent this procedure.

1.2. Precautions/Limitations:

1.2.1. Evidence

1.2.1.1. Liquid items such as blood standards shall be refrigerated not frozen. Stained materials may be frozen, refrigerated or stored at room temperature in a dry environment. Items to be analyzed for latent prints should not be frozen or refrigerated.

1.2.1.2. Stains that are still moist shall be air dried in a secure area before submission to the laboratory.

1.2.1.3. Clean paper or cardboard containers shall be used to package dried evidence. Plastic containers shall not be used for packaging because they hold moisture which often leads to putrefaction of biological materials. Exceptions to this rule would include blood tubes, body tissue samples and condoms. Each item should be placed in separate containers from the point of collection.

1.2.1.4. If multiple items are placed in one container at time of collection, they may be submitted in the single, original package.

1.2.1.5. Specific examinations performed on items of serological evidence shall be determined by laboratory personnel.

1.3. Related Information:

1.3.1. Appendix 1 Flow Charts

1.3.2. Work Sheet Manual

1.4. Instruments:

1.4.1. Balances – An analytical balance used for preparation of analytical reagents and buffers.

1.4.2. Centrifuges – A serofuge capable of operating at 3400 rpm and an ultra-centrifuge capable of up to 15,000 rpm, are used for separation of solid components from fluids.

1.4.3. Alternate light source (ALS) – A light source to aid in the location of stains and trace evidence by use of various wavelengths of light.

1.4.4. Crosslinker - Preprogrammed ultraviolet exposure unit which is factory set to ~120,000 microjoules per cm^2.

1.4.5. Digital camera – Used to document evidence and/or the packaging.

1.4.6. Microscopes – A light microscope with magnification up to 400X, a stereoscope for general screening of items, and a phase contrast microscope for the identification of spermatozoa.

1.4.7. Miscellaneous Laboratory Equipment – Supportive laboratory equipment including ovens, incubators, pipettors, rotators, stirring/heating plates, vortex mixers, vacuum pumps, UV lights, and refrigerators/freezers for storing of reagents, buffers and evidence.

1.4.8. pH meter – An instrument capable of manual or automatic temperature compensation and reading +/- 0.01 pH units. Used for preparation of buffers and reagents.

1.5. Reagents/Materials: See Reagent Preparation Manual for instructions and logs. (Reagent Prep Manual)

1.5.1. Alpha Naphthyl Phosphate Solution
1.5.2. Amylase Buffer
1.5.3. Amylase Plates
1.5.4. Acid phosphatase Acetate Buffer
1.5.5. Acid phosphatase Dye Solution

1.5.6. Coomassie Blue Stain
1.5.7. Destain
1.5.8. Florence Iodine Solution (for Amylase)
1.5.9. Glucose, saturated
1.5.10. 0.176M H_2O_2 (Luminol Solution B)
1.5.11. 3% H_2O_2
1.5.12. 0.004M Luminol (Luminol Solution C)
1.5.13. Phenolphthalein Stock Solution
1.5.14. Phenolphthalein Working Solution
1.5.15. 0.4N Sodium Hydroxide (Luminol Solution A)
1.5.16. 10% Sodium Hydroxide
1.5.17. Species Origin Ouchterlony Plates
1.5.18. Takayama Solution

1.6. Hazards/Safety:

1.6.1. All chemicals shall be handled in a safe method as referenced in the specific MSDS.

1.6.2. Preparation of the Phenolphthalein Stock Solution shall be performed in a chemical fume hood.

1.6.3. The spraying of Luminol reagent in the laboratory shall be confined to a chemical fume hood whenever practical.

1.6.4. Universal Precautions shall be in use whenever biological materials are being handled.

1.6.5. Extreme caution shall be used when handling liquid body fluid samples.

1.6.6. Biological waste shall be disposed of in the appropriate waste receptacle.

1.7 Reference Materials/Controls/Calibration Checks:

1.7.1 All commercial antisera are tested for accuracy and specificity before use in casework.

1.7.1.1 Each new lot of anti-human antisera shall be analyzed against various animal sera and human blood to confirm specificity. Animal sera shall include but not be limited to deer, dog, cat, cow, chicken, monkey, swine, and the species used to produce the antiserum (goat for goat anti-human; rabbit for rabbit anti-human). The worksheet used to record the results shall be retained.

1.7.1.2 Each new lot of animal antisera shall be analyzed against human blood and any available related species to confirm specificity. The worksheet used to record the results shall be retained.

1.7.2 Running known controls with each test ensures the accuracy and specificity of the test results. See the specific test for the appropriate controls to be run.

1.8. Procedures/Instructions:

1.8.1. Examination Procedure

1.8.1.1. Standards for Comparison

1.8.1.1.1. Appropriate standards, such as whole blood or buccal swabs, from those individuals involved should be submitted along with the questioned materials to be analyzed.

1.8.1.1.2. The whole blood standard should be submitted in a purple top tube that contains ethylene-diamine-tetra-acetic acid (EDTA). Prior to examination, while in the custody of the ISP Laboratory, this tube shall be refrigerated.

1.8.1.1.3. A stain card shall be prepared from at least one whole blood standard from each individual and shall be retained by the laboratory for future reference and analysis.

1.8.1.1.4. Buccal swab standards shall be retained by the laboratory for future reference and analysis.

1.8.1.1.5. The analyst's notes shall indicate the presence of hair standards.

1.8.1.1.6. In cases where a secondary standard is needed, the item to be used as a secondary standard shall be clearly identified as such.

1.8.1.1.6.1. The use of a blood stained article of clothing may be appropriate if stains from the person wearing the clothing can be definitively identified (i.e. blood from around the wound area).

1.8.1.1.6.2. In the case of a missing person, personal items such as tooth brush, hair brush, razor or other item may be submitted.

1.8.1.1.6.3. When a contributor requests items to be used as secondary standards during the submission process, it should be indicated on the Request for Laboratory Examination form.

1.8.1.2. Trace Evidence

1.8.1.2.1. A clean piece of paper shall be used under each item as it is examined. The exam paper should be retained in the original package. After returning the item to its package, fold the exam paper, tape it closed (making sure all trace evidence is secure within the bundle) and place it in the package with the item.

1.8.1.2.2. Items may be tape lifted to collect evidence. Where tape lifting is not appropriate, tweezing and/or scraping may be substituted. Items involving multiple examination requests shall only be tape lifted or scraped after consultation with analysts from the other disciplines.

1.8.1.2.3. All collected trace evidence shall be stored with original item unless value is immediately apparent.

1.8.1.2.4. Analyst shall document in the notes the presence of hair, fibers, plant material, glass, paint, etc. Such trace evidence shall be reported out only when it is deemed to have value. In those cases where the material appears to be extraneous debris of no significance, the trace statement may be omitted from the report.

1.8.1.2.5. The approximate number and quality of hairs shall be noted for hair combings.

1.8.1.2.6. In cases where there is other DNA evidence of investigative value that has been tested and profiles obtained, no DNA testing of the collected hair shall be performed without the approval of a Biology Unit Supervisor or Laboratory Manager.

1.8.1.2.7. In cases where there is no other DNA evidence of value, the prosecutor/investigator shall be contacted for approval of consuming the hair sample before further analysis of the hair is performed.

1.8.1.2.8. When appropriate, examination of the hair collected shall be performed to determine if hair consistent with human origin is present.

1.8.1.2.9. Human hairs can be submitted for DNA analysis when beneficial.

1.8.1.3. Dried Stains (See Examination flow charts)

1.8.1.3.1. Visual Inspection – A visual inspection of the item shall be conducted to determine the location, if present, of questioned stains. An ultraviolet light or an alternate light source may be used to help locate stains.

1.8.1.3.2. Stain Identification – If a sample is being retained because of a positive result, (phenolphthalein, acid phosphatase, or confirmatory testing) then amylase testing need not be performed.

1.8.1.3.3. If phenolphthalein is being performed on stains which demonstrated positive luminol result and phenolphthalein is negative, no further testing should be performed due to the possibility of false positives for luminol. These results should be reported out as inconclusive.

1.8.1.4. Stain Evaluation – After attempting to identify the stain, the stain shall be evaluated for further testing.

1.8.1.4.1. If no biological material is identified on the item, no further testing shall be performed, except for instances where touch DNA or handler/wearer analysis is appropriate.

1.8.1.4.2. If the stain is found to be biological but no standards are available for comparison, the stain or a portion thereof shall be retained by the laboratory for future testing. DNA testing may be performed and results compared to CODIS when eligible.

1.8.1.4.3. If the stain is found to be biological material and all necessary standards have been submitted, the analyst shall determine if DNA typing shall be performed based on an evaluation of the stain. Things that may be considered include, but are not limited to, the amount,

size, concentration, location and condition of the stain.

1.8.2. Phenolphthalein (Kastle-Meyer Reagent), Presumptive Test for Blood.

1.8.2.1. Principle: Phenolphthalein is an oxidizable organic molecule, which can be oxidized by free hydroxyl ions liberated by peroxidase-like action. The heme group of hemoglobin possesses a peroxidase-like activity, which may catalyze the breakdown of hydrogen peroxide to form free hydroxyl radicals. Phenolphthalin (reduced form) is oxidized by the free hydroxyl ions to phenolphthalein (oxidized form), producing a pink color.

Phenolphthalein is a presumptive test for the presence of blood. Confirmatory testing shall be performed for conclusive identification. Phenolphthalein has been shown to give false positives or weak reactions with various oxidizing agents, plant material, etc.

1.8.2.2. Procedure

1.8.2.2.1. Rub the suspected bloodstain with a piece of filter paper or a cotton swab or make a small cutting of the suspected bloodstain and place on white filter paper or in a white spot plate well.

1.8.2.2.2. Add one to three drops of the phenolphthalein working solution to the stain.

1.8.2.2.3. Wait 10-15 seconds, assuring no pink color develops at this time.

1.8.2.2.4. Add one to three drops of 3% Hydrogen Peroxide.

1.8.2.2.5. Upon addition of the Hydrogen Peroxide an immediate pink color is indicative of the possible presence of blood.

1.8.2.3. Reagents shall be tested with positive and negative controls and the results recorded in the case notes. This shall be performed and recorded daily when in use and prior to use on evidence. Lot numbers and/or preparation dates of reagents shall also be recorded.

1.8.3. Luminol, Presumptive Test for Blood.

1.8.3.1. **Principle**: Luminol is a presumptive test designed to process large areas where stains are not readily visible. Luminol works best on old stains (where the heme group has been converted to hemin) and while it does soak the area tested, it should not interfere with additional testing. In the luminol test, the hemin acts as a catalyst, triggering the oxidation of luminol by hydrogen peroxide in an alkaline solution.

Confirmatory testing shall be performed for conclusive identification. Luminol has been shown to give false positives with certain metal compounds, plant peroxidases and some cleansers, especially cleansers which contain hypochlorite (bleach). Luminol is an alternative method that can be used on phenolphthalein negative items where bloodstains are not easily visible.

1.8.3.2. Procedure

1.8.3.2.1. Prepare working solution by combining 10 ml Solution A, 10 ml Solution B, 10 ml Solution C and 70 ml distilled water. Alternatively, using previously prepared spray bottle, fill to marks for each reagent.

1.8.3.2.2. In the dark, test a piece of known blood-stained sample for positive reaction before using on evidence or at a crime scene.

1.8.3.2.3. All individuals present during testing shall wear gloves, eye protection and a surgical mask or equivalent.

1.8.3.2.4. Spray the area of interest in the dark until well-soaked. Luminol's reaction with a true bloodstain produces a luminescent glow, frequently in patterns such as spatters, smears, wipes or drag marks, or even footwear impressions.

1.8.3.3. Alternate Procedure

1.8.3.3.1. Prepare the luminol solution by adding the contents of the powdered luminol tube (8 oz size) to 8 oz (250 ml) of distilled water in a plastic reagent bottle. Alternate powdered luminol tubes are available in 4 oz and 16 oz sizes. If these are used, add the corresponding amount of distilled water, 4 oz (125 ml) or 16 oz (500 ml).

1.8.3.3.2. Gently mix the powder and distilled water with a gentle swirling action in order to avoid mixing an excessive amount of air or oxygen into the solution.

1.8.3.3.3. Transfer the luminol solution into a fine mist spray bottle. Any unmixed powder remnants should be left in the bottom of the plastic reagent bottle and not transferred into the spray bottle to avoid clogging the sprayer.

1.8.3.3.4. The mixed luminol solution should be used within about 20 - 30 minutes.

1.8.3.3.5. Follow steps 1.8.3.2.2 through 1.8.3.2.4 as above.

1.8.3.4. Any remaining luminol solution cannot be stored for later use because the reagent will lose potency. The remaining luminol solution can be disposed by washing it into the sink with a large quantity of water. Also, be sure to clean out the spray bottle and nozzle with clean water, as any luminol remaining in the sprayer will form a difficult-to-remove clog.

1.8.3.5. Reagents shall be tested with positive and negative controls and the results recorded in the case notes. This shall be performed and recorded daily when in use and prior to use on evidence. Lot numbers and/or preparation dates of reagents shall also be recorded.

1.8.4. Takayama; Confirmatory Test for Blood

1.8.4.1. Principle: Takayama reacts with the heme portion of the blood to form characteristic hemochromogen microcrystals.

Some vegetable peroxidases may form Takayama crystals similar to those formed by blood; however, these substances do not have the same appearance or physical properties as blood.

1.8.4.2. Procedure

1.8.4.2.1. Prepare working solution by combining glucose, sodium hydroxide, pyridine, and distilled water. The working solution may be made up and stored in the refrigerator for up to one month.

1.8.4.2.2. Place a small cutting, few threads or flakes on a microscope slide and cover with a cover slip. Alternatively, a drop of stain extract may be dried onto the slide.

1.8.4.2.3. Flood under the cover slip with the prepared Takayama reagent.

1.8.4.2.4. Gently heat the microscope slide on a hot plate or equivalent heat source. Optionally, if the stain is weak, the test may be allowed to sit at room temperature.

1.8.4.2.5. When cutting turns bright pink/red, remove from heat and allow to cool to room temperature before observing under the microscope. Negative samples may never turn to the bright pink color.

1.8.4.2.6. Bright red, spiky crystals should be apparent in the presence of a sufficient concentration of blood. Tapping of the cover slip to separate crystals from the cutting may be necessary to observe crystals.

1.8.4.3. Reagents shall be tested with positive and negative controls and the results recorded in the case notes. This shall be performed and recorded daily when in use and prior to use on evidence. Lot numbers and/or preparation dates of reagents shall also be recorded.

1.8.5. Species/Human Origin using Ouchterlony Plates

1.8.5.1. **Principle**: Human anti-serum reacts with proteins in human blood and some tissues to form a visible white precipitate band.

1.8.5.2. **Procedure**

1.8.5.2.1. In previously prepared Ouchterlony plates, cut wells as illustrated in the diagram below. The size of the wells can vary from 2-6 mm in diameter, with about 5 mm between wells. Remove the agar plugs.

1.8.5.2.2. In the known well, place either a cutting from a known human bloodstain and fill the well with distilled water; or fill the well with liquid human serum or blood.

1.8.5.2.3. In the blank well, place the negative control/background. Distilled water shall be included as a negative control at least once in each series of tests.

1.8.5.2.4. In the two sample wells, place a small cutting and fill the well with distilled water. Alternatively, a cutting of the stain can be eluted in a microtube in distilled water. The extract is then placed in the well. While preferable to run in duplicate, limited sample size may require only one test.

1.8.5.2.5. In the center well, place the human anti-serum.

1.8.5.2.6. Allow to stand overnight at room temperature.

1.8.5.2.7. Observe white precipitate bands present for each test, by placing a light beneath the plate.

1.8.5.2.8. Alternatively, a small amount of Coomassie blue stain can be poured on the plate, drained, and rinsed off with water or use destain to aid the visualization of bands.

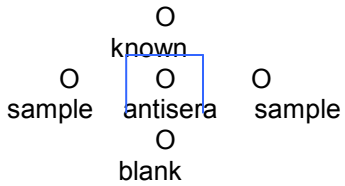
1.8.5.2.9. The white precipitate bands shall form a box type configuration to indicate an identity reaction.

1.8.5.2.10. Manufacturer's instructions shall be followed in preparation of antisera. Antisera may be aliquoted, frozen, or refrigerated.

1.8.5.3. **Procedure for Other Species**

1.8.5.3.1. In place of known human serum, appropriate animal serum is placed in known well.

1.8.5.3.2. Antiserum of the species of interest is placed in the center well.



1.8.5.4 Reagents shall be tested with positive and negative controls and the results recorded in the case notes. This shall be performed and recorded

daily when in use. Lot numbers and/or preparation dates of reagents shall also be recorded.

1.8.6. Acid Phosphatase, Presumptive Test for Semen

1.8.6.1. **Principle:** Seminal fluid is typically found to contain acid phosphatase in levels 400 -1000 times those found in other body fluids. This test uses the ability of acid phosphatase to hydrolyze a phosphate ester substrate to release a free alcohol, which in turn reacts with a diazonium salt to give a brilliantly colored end product.

Acid Phosphatase is a presumptive test for the presence of semen. Confirmatory testing shall be performed for conclusive identification. Possible sources of acid phosphatase other than semen include vaginal secretions, urine, sweat or fecal material.

1.8.6.2. **Procedure:** Prepare substrate and dye solution immediately prior to testing. These solutions will be stable for 1-2 hours at room temperature. Alternatively, use previously aliquoted and frozen solutions.

1.8.6.2.1. Weigh out about 20 µg of alpha naphthyl phosphate and place in shell vial or small test tube.

1.8.6.2.2. Weigh out about 20 µg of fast blue or fast red dye (shall be in diazonium salt form) and place in second shell vial or small test tube.

1.8.6.2.3. Add about 1 ml of acetate buffer to each and mix.

1.8.6.2.4. Cut a small (approximately 2 mm x 2 mm) portion of the stained material and place it on a small piece of filter paper, in a white ceramic spot plate well or in a small test tube. An extract can be used.

1.8.6.2.5. Wet the cutting with the alpha naphthyl phosphate solution. Wait about 1 minute.

1.8.6.2.6. Add 1-2 drops of the dye solution to the cutting.

1.8.6.2.7. The development of color (depending on dye used) in less than 15 seconds is indicative of the presence of acid phosphatase. Color soaked into the filter paper under the sample is also indicative of a positive test.

1.8.6.2.8. A slow development of color could be an indication of acid phosphatase from sources other than semen. This may be indicated on the worksheet as a slight color change (SCC).

1.8.6.2.9. The interpretation of the results shall be reported by the analyst.

1.8.6.3. Reagents shall be tested with positive and negative controls and the results recorded in the case notes. This shall be performed and recorded daily when in use and prior to use on evidence. Lot numbers and/or preparation dates of reagents shall also be recorded.

1.8.7. Extraction of Body Fluid Stains

1.8.7.1. Principle: Evidentiary stains can be extracted and the extracts used for body fluid stain identification.

1.8.7.2. Procedure:

1.8.7.2.1. Prepare samples (and backgrounds, if used) for testing by eluting a cutting of the stain (approximately 3 mm by 3 mm) in an appropriate amount of distilled water in a microcentrifuge tube overnight in the refrigerator. Alternatively the sample can be eluted in a 37°C oven for a minimum of 30 minutes or at room temperature for a minimum of 1 hour.

1.8.7.2.2. After incubation, the cutting should be removed, placed into the cap with holes for drainage or a spinex basket, and the extract spun down.

1.8.8. Visual Observation of Spermatozoa

1.8.8.1. Principle:

The visual identification of spermatozoa is a scientifically accepted method of positively identifying the presence of seminal material.

1.8.8.2. Procedure:

1.8.8.2.1. Place ~10 µl of the previously extracted sample on a microscope slide and cover. Phase contrast microscopy is used to differentiate the cellular appearance. Spermatozoa are round to ovoid with an acrosomal cap, midpiece, and tail. When the tail and midpiece are not present, the distinctive flattened profile of turned spermatozoa should be observed.

1.8.8.2.2. Alternatively a small (1 mm x 1 mm) cutting may be eluted directly onto the slide with a drop of distilled water, agitating with forceps. Remove the cutting and cover the extract with a cover slip.

1.8.8.2.3. The observations should be rated using the following system:

1.8.8.2.3.1. POS (+) Only a few sperm or sperm heads on the entire slide.

1.8.8.2.3.2. 1+ One sperm or sperm head per several fields of view.

1.8.8.2.3.3. 2+ One sperm or sperm head in about half of all fields of view.

1.8.8.2.3.4. 3+ One sperm or sperm head in most fields of view.

1.8.8.2.3.5. 4+ Several sperm or sperm heads in most fields of view.

1.8.9. Christmas Tree Stain (Kernechtrot-Picroindigocarmine Differential Stain)
(Optional procedure to help visualize spermatozoa)

1.8.9.1. Principle: The Christmas tree stain is used to assist in scanning slides for the presence of spermatozoa. Red and green colored stains are utilized to enhance the appearance of spermatozoa and epithelial cells in biological samples.

1.8.9.2. Procedure

1.8.9.2.1. Pipette ~5-10 µl of the pellet from the bottom of extracted sample tube and place onto microscope slide. Previously prepared slides from the evidence collection kit may go directly to heat fix and staining procedures.

1.8.9.2.2. Allow sample to air dry or dry in a 56°C oven.

1.8.9.2.3. Heat fix the sample onto the slide.

1.8.9.2.4. After the slide has cooled, place on a level surface and add several drops of the SERI Christmas Tree Stain A (red stain) to the slide. Stain for about 15 minutes.

1.8.9.2.5. Rinse stain from the slide by gently running distilled water over the slide.

1.8.9.2.6. Return slide to a level surface and add several drops of the SERI Christmas Tree Stain B (green stain) to the slide. Stain for about 10 seconds.

1.8.9.2.7. Rinse stain from the slide by gently running 95 to 100% ethanol over the slide.

1.8.9.2.8. Allow slide to air dry or dry in 56°C oven. After drying, slide is ready to view microscopically.

1.8.9.3. Interpretation

1.8.9.3.1. Spermatozoa will appear as small red/pink structures and have the following staining characteristics:

Acrosomal cap	clear to pink
Post acrosomal cap	dark pink to red
Midpiece	green to blue
Tail	green to blue

1.8.9.3.1.1. Nucleated epithelial cells will appear as green rhomboid-like structures with a red/pink nucleus. The cell's appearance can vary in shape, often being folded or overlapping and are normally larger than the spermatozoa heads.

1.8.9.3.2. **NOTE**: The smear slides contained in evidence collection kits shall be viewed using phase contrast microscopy before proceeding to the Christmas tree stain procedure.

1.8.10 Amylase

1.8.10.1 Principle:

1.8.10.1.1 The detection of amylase in forensic stains is of particular importance to the analysis of cases involving oral intercourse. Performing amylase testing in sexual assault cases will be dependent on other results and the needs of the specific case. Elevated amylase levels may be from oral intercourse or from elevated levels within the vaginal cavity. The amount of amylase on items such as cigarette butts, envelopes, stamps, masks, etc. is not a good indication of the amount of cellular material available for DNA analysis.

1.8.10.1.2 Amylase is an enzyme which is responsible for the hydrolysis of starch (amylose and amylopectin) to glucose and maltose. Amylase is present in high concentrations in saliva, pancreatic fluid, and in fecal material. Limited amylase activity is also present in other body fluids.

1.8.10.2 Procedure

1.8.10.2.1 In previously prepared plates, cut wells, 2-6 mm in diameter, in a circle around the outer edge of the plate (not to exceed 7 wells). One additional well can be cut into the center. Remove the agar plugs.

1.8.10.2.2 In one well, place a minimum of 5 µl of a prepared extract of a known dried saliva stain or a 1:100 dilution of liquid saliva in distilled water.

1.8.10.2.3 In succeeding wells, place the same amount of extracted samples. It is important to use the same volume for knowns and unknowns.

1.8.10.2.4 In the last well, place distilled water or a prepared extract of a blank substrate.

1.8.10.2.5 Incubate at 37°C for 18 to 24 hours.

1.8.10.2.6 Stain with Florence iodine diluted ~1:100.

1.8.10.2.7 Measure area not stained by iodine across the diameter and record.

1.8.10.2.8 A questioned sample should be reported positive for amylase and retained for DNA analysis if the diameter is 70% or greater than the diameter of the known saliva sample. A sample with a result of less than 70% may be retained at analyst's discretion. To say "no amylase detected," the result shall be consistent with the negative control.

1.8.10.2.9 The interpretation of the results shall be reported by the analyst.

1.8.10.3 Reagents shall be tested with positive and negative controls and the results recorded in the case notes. This shall be performed and recorded daily when in use. Lot numbers and/or preparation dates of reagents shall also be recorded.

1.8.11 Visual Observation of Human Hair

1.8.11.1 Principle: Before DNA analysis can be performed on hair collected as evidence, a determination of possible human origin and the identification of the root shall be performed.

1.8.11.2 Procedure

1.8.11.2.1 When the questioned hairs are on a tape lift, the lift should be examined using a stereomicroscope to eliminate obvious hair fragments, fibers, and animal hairs. All hairs of possible human origin with sufficient root material should be removed from the lift and placed in a paper bindle. A wet mount should be prepared when further examination is required to determine human origin and/or presence of root material. Questioned hairs are placed on a microscope slide with a drop of distilled water, covered with a cover slip, and examined with a light microscope. The number and general appearance of the human hairs should be noted when practical. If additional hairs/fibers are present in the original package, they should be placed in a separate bindle and retained in the package.

1.8.11.2.2 Observation should begin with the presence of scales (imbricate in humans and some animals). Human hairs demonstrate a wide variety in color and pigment distribution among individuals. The color in the cortex should be fairly uniform throughout the length and breadth of the hair. An exception would be dyed or bleached hairs, which could demonstrate a sudden or gradual color change at the dye line. (Animal guard hairs demonstrate a characteristic color banding). The medulla (the amorphous central core) can be continuous, discontinuous, fragmentary, or not present in human hair. Medullary index should be one-third or less the total diameter of the hair. There is considerable variation in the general appearance of human hairs depending on the body area. In general there is less variation in diameter along the length in human hairs than in animal hairs. The appearance of the root is very important both in determining origin and in determining the area to sample for DNA. The appearance of a large skin tag is not necessarily an indication of success in extraction of DNA.

1.8.11.2.3 Examination documentation shall be of sufficient detail so that an equally qualified analyst is provided enough information in the case notes so as to reach the same conclusion. In the case of hair, when a “human hair” conclusion is reached the notes shall include observations regarding scale type, medulla, coloring/pigment distribution and any other pertinent observations.

1.8.12 Crosslinker Procedure

Consumables (pipette tips, microtubes, columns, etc…) may be exposed in the crosslinker for 2 to 15 minutes before use in casework. Time in excess of 15 minutes shall have the approval of the Technical Leader documented in the case record.

The use of the crosslinker may reduce the presence of amplifiable DNA.

1.9. Records:

1.9.1 Examination of Sexual Assault kits and Suspect Collection kits shall be recorded on the appropriate worksheets.

1.9.2 Results of testing of body fluids shall be recorded on the worksheets designed for that purpose, or alternatively be recorded in the case notes within the description of the item as long as all required controls are documented appropriately.

1.9.3 All photos and electronically created documentation shall be stored as part of the case record. See Appendix 5 for instructions.

1.9.4 The technical review of the case record shall be recorded on the worksheet provided for that purpose.

1.10. Interpretations of Results:

1.10.1. The quality and quantity of a sample shall determine which tests are performed. Conclusions reached are based on the actual tests performed in each case. See specific tests for interpretation guidelines.

1.10.2. The analyst shall report out any identified body fluid stains present and list all items retained for possible DNA analysis in the report.

1.11 Report Writing:

(required) {options} [example]

Wording Where Only Presumptive Testing Positive

Presumptive testing indicated the possible presence of {seminal material/blood} on X (item #). Additional serological testing did not confirm the presence of {seminal material/blood}.

Or When Sample Size Is Limited

Presumptive testing indicated the possible presence of {seminal material/blood} on X (item #). No further serological testing was performed in order to preserve the sample for DNA analysis.

Or When Not of Value

Presumptive testing indicated the possible presence of {seminal material/blood} on X (item #). No further serological testing was performed at this time. {If further analysis is desired, please contact the reporting analyst.}

The {oral swab standard/red topped whole blood standard} contained in item X was not {retained/examined} due to the presence of multiple standards.

Negative Presumptive Testing

No {blood/seminal material/amylase} was detected on X (item #).

No Testing Performed

No stains were observed on X (item #) for serological testing.

No serological testing was performed on X (item #).

Wording For Confirmatory Testing

Blood was detected on X (item #).

Semen/Seminal material was detected…

Wording For Amylase

When >70% of known or if less than 70% and retained (analyst's discretion):
Amylase was detected on X (item #).

When <70% and not retained (analyst's discretion):

X (item #) failed to demonstrate a sufficient quantity of amylase for further analysis.

Wording For Trace Evidence

Potential trace evidence was {observed on/collected from} X (item #). Trace evidence may include but is not limited to paint, glass, hair, and fibers. To determine if further examination is warranted, please contact the reporting analyst.

Wording For the Identification Of Human Hair

Hair, which demonstrated characteristics consistent with human hair and has root material with the potential for nuclear DNA analysis, was {present in/collected from} X (item #).

Hair, which demonstrated characteristics consistent with human hair, but does not have root material with the potential for nuclear DNA analysis, was {present in/collected from} X (item #). To determine if further analysis is beneficial, please contact the reporting analyst.

Hair, which demonstrated characteristics not consistent with human hair, was present in X (item #).

Wording For Standards

A stain card was prepared from the blood standard of [John Smith] (item #).

A stain card was prepared from the blood standard of [Jane Smith] (item #) contained in item X.

A {buccal swab/hair/other} standard of [John Smith] was present in item #.

Request For Standards

If additional information is desired, please submit an appropriate DNA standard (such as a blood standard in a purple top tube or an oral swab standard) from [victim's name] {and/or} any suspect{s}.

Retention Statements - Serology or Both Reports
Note: Retention statements may be in paragraph form if they contain 3 or fewer items. Item numbers should contain all leading zeros.

No Further Analysis To Be Performed – e.g. Stain Cards Only, Nothing Going On To DNA.

The following items were retained by the Indiana State Police Laboratory for the possibility of future analysis:

A Stain card
B Stain card

Going On To DNA Analysis

The following items were retained by the Indiana State Police Laboratory for DNA analysis: {"for DNA analysis" is not to be used in 'Both' reports}
(multiple columns optional)

A [swab]
B [swab]
C [cutting]
D [etc.]
X [etc.]
Y [stain card]
Z [stain card]

Original Items

The [blood standard] (item XA), and the [vaginal wash] (item XB) returned to item X; the [jeans] (item Y); and the original packaging for item Z will be returned to the submitting agency.

When completed, results of any DNA analysis will be provided in a separate report. All qualifying DNA profiles will be entered into the Indiana DNA database and searched on a routine basis.

Request for Examination Withdrawn

The request for {DNA} analysis was withdrawn by John Smith, [Anywhere Police Department], on [date].

Note: Report wording may be altered with the approval of a Biology Unit Supervisor.

1.12 References:

1.12.1 Forensic Science Handbook, ed. Richard Saferstein. 1982. Prentice-Hall. New Jersey.

1.12.2 Forensic Science Handbook, Volume I. ed. Richard Saferstein. 2002. Prentice-Hall. New Jersey.

1.12.3 Forensic Science Handbook, Volume II. ed. Richard Saferstein. 1988. Prentice-Hall. New Jersey.

1.12.4 Forensic Science Handbook, Volume III. ed. Richard Saferstein. 1993. Prentice-Hall. New Jersey.

1.12.5 Gaensslen, R.E. 1983. Sourcebook of Forensic Serology, Immunology and Biochemistry. United States Government Printing Office, Washington D.C.

1.12.6 Jungreis, Ervin. 1997. Spot Testing Analysis. John Wiley & Sons, New York.

1.12.7 Laux, D. L. 1992. Detection of bloodstains; A Training Manual. Richfield Ohio.

1.12.8 Jeyendran, R. S. 2000. Interpretation of Semen Analysis Results, a Practical Guide. Press Syndicate of the University of Cambridge, Cambridge, United Kingdom.

1.12.9 Deedrick, D. W. and Koch, S. L. Microscopy of Hair, Part 1: A Practical Guide and Manual for Human Hairs. *Forensic Science Communications*. January 2004. Volume 6(1).

1.12.10 Deedrick, D. W. and Koch, S. L. Microscopy of Hair, Part 2: A Practical Guide and Manual for Animal Hairs. *Forensic Science Communications*. July 2004. Volume 6(3).

1.12.11 Practical Aspects of Rape Investigation: A Multi Disciplinary Approach. Third Edition. eds. Robert R. Hazelwood and Ann Wolbert Burgess. Elsevier, New York.

1.12.12 Saferstein, R. 1995. Criminalistics, An Introduction to Forensic Science. Fifth Edition. Prentice Hall, New Jersey.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

2.0 DNA METHODS:

2.1 Scope:

This test method is designed for the guidance of Laboratory personnel who assist investigations where the specificity of DNA testing is needed. Evidence amenable to DNA testing includes blood, seminal fluid, saliva, bone, tissue and hair. This manual may be expanded or altered as new techniques and/or genetic systems are found applicable.

2.1.1 The DNA is first extracted from the biological sample, quantified, amplified to produce many fluorescently tagged copies of specific regions of the DNA and finally processed to separate and detect a DNA profile(s). The analyst compares the DNA profile identified in the sample with the DNA profile from a known standard from an individual to determine similarities or dissimilarities. In addition, the analyst may provide an estimate of occurrence of the genetic profile in the population.

2.1.2 Return of Evidence

2.1.2.1 Stain cards from whole blood samples, cuttings from body fluid stains, DNA extracts, and other selected DNA evidence shall be retained by the laboratory for any possible future testing. For amplified DNA see 2.8.1.2.6. The retained items may be released at the prosecutor's request. The request shall be documented by the analyst in the case record.

2.1.2.2 A valid court order shall circumvent this procedure.

2.2 Precautions/Limitations:

2.2.1. Evidence

2.2.1.1 Liquid items such as blood standards shall be refrigerated.

2.2.1.2 Stains which are still moist shall be air dried in a secure area before submission to the Laboratory.

2.2.1.3 Clean paper or cardboard containers shall be used to package dried evidence. Plastic containers shall not be used for packaging because they hold moisture which often leads to putrefaction of biological materials. Exceptions to this rule would include blood tubes, body tissue samples and condoms.

2.2.1.4 Specific examinations performed on items for DNA analysis shall be determined by laboratory personnel.

2.3 Related Information:

2.3.1 Worksheet Manual

2.3.2 Appendix 2 Mixture Interpretation Flow Chart

2.4 Instruments:

2.4.1 Applied Biosystems™ 3130 Genetic Analyzer Capillary Electrophoresis Instrument – Simultaneously separates and detects multiple amplified DNA samples by size by capillary electrophoresis using fluorescent tagged primers.

2.4.2 Applied Biosystems™ 7500 Real Time PCR System – Measures accumulation of amplification product over time to determine total DNA present by the use of a fluorescent reporter molecule.

2.4.3 Autoclave - An instrument that uses heat and steam to sterilize solutions, contaminated equipment and waste.

2.4.4 Balance - An analytical balance used for preparation of analytical reagents and buffers.

2.4.5 BIOMEK NX^{P} Automation Platform – Robotic liquid handling system.

2.4.6 BIOMEK 3000 Automation Platform – Robotic liquid handling system.

2.4.7 Centrifuges - A microcentrifuge capable of operating at greater than 15,000 rcf and a clinical serofuge capable of operating at a maximum of 3,400 rpm; used for the purification of DNA in evidence samples and the separation of solid components from liquids, respectively.

2.4.8 Crosslinker - Preprogrammed ultraviolet exposure is factory set to 120,000 microjoules per cm^2. Preset ultraviolet time exposure is factory set to 2 minutes.

2.4.9 Laminar Flow Hood – An air purifying biohazard cabinet that maintains a nominal inflow velocity of 80 fpm which prevents contaminants from entering or escaping the work area.

2.4.10 Maxwell® 16 – Robotic extraction system.

2.4.11 Microscope - A light microscope with magnification up to 400X, a stereoscope for general screening of items, and a phase contrast microscope for the identification of spermatozoa.

2.4.12 Miscellaneous Laboratory Equipment - Supportive laboratory equipment consisting of ovens, incubators, rotators, pipettors, water baths, stirring/heating plates, vortex mixers, visible/UV light boxes, microwave, thermometers, temperature verification system, vacuum pump, refrigerators/freezers for storing of reagents, buffers and evidence.

2.4.13 pH Meter – An instrument capable of manual or automatic temperature compensation and reading +/- 0.01 pH units. Used for preparation of buffers and reagents.

2.4.14 Thermal cycler - An instrument that can be programmed to rapidly cycle between high and low temperatures. This process is used to make many fluorescently tagged copies of specific regions on a DNA strand(s).

2.4.15 Water Purification System - An apparatus that routes water through a series of filtering devices to produce high quality, uncontaminated water used in buffer preparation and DNA typing methods.

2.5 Reagents/Materials: See Reagent Preparation Manual for instructions and logs where appropriate. (Reagent Prep Manual**)**. Reagents critical to the DNA analysis process are listed in the Critical Reagent Manual.

2.5.1 Digest/Wash Buffer
2.5.2 DTT 0.39M
2.5.3 DTT 1M
2.5.4 EDTA 0.5M
2.5.5 Nuclease Free Water (NFH2O)
2.5.6 Proteinase K (Pro K) 10 mg/ml
2.5.7 Proteinase K (Pro K) 18 mg/ml
2.5.8 Sarkosyl 20% w/v
2.5.9 SDS 20% w/v
2.5.10 Stain Extraction Buffer Stock Solution
2.5.11 Stain Extraction Buffer with DTT
2.5.12 Stain Extraction Buffer for Automation
2.5.13 TE^{-4}
2.5.14 TRIS/EDTA/NaCl Solution
2.5.15 TRIS-HCl pH 7.5, 1M
2.5.16 TRIS-HCl pH 8.0, 1M

2.6 Hazards/Safety:

2.6.1. All chemicals shall be handled in a safe method as referenced in the specific Material Safety Data Sheets (MSDS).

2.6.2. The addition of phenol/chloroform/isoamyl alcohol during extraction, vortexing of the extracts, and transfer to Microcons® shall be done in a chemical fume hood.

2.6.3. The manual preparation of samples for electrophoresis by the addition of Hi-Di™ formamide shall be confined to a chemical fume hood. **Caution:** Formamide is an irritant and teratogen; therefore universal precautions and a fume hood shall be utilized when manually working with formamide to avoid inhalation and contact with the skin.

2.6.4 The manual handling of lysis buffer should be confined to a chemical fume hood.

2.6.5. Universal Precautions shall be in use whenever biological materials are being handled.

2.6.6. Extreme caution shall be used when handling liquid body fluid samples.

2.6.7. Biological waste shall be disposed of in the appropriate waste receptacle.

2.7 Reference Materials/Controls/Calibration Checks:

2.7.1 The accuracy and specificity of test results are ensured by running known DNA controls and reagent controls at the same time as evidence samples. See the specific test for the appropriate controls to be run and the interpretation of the results.

2.8 Procedures/Instructions

2.8.1 Examination Procedure

2.8.1.1 Standards for Comparison

2.8.1.1.1 Appropriate standards, preferably whole blood or buccal swabs, from individuals involved, should be submitted along with the questioned materials.

2.8.1.1. 2 Whole blood standards should be submitted in a purple top tube that contains ethylene-diamine-tetra-acetic acid (EDTA). This tube shall be refrigerated.

2.8.1.1.3 Alternately a swabbing taken from the inside cheek of an individual may be collected, dried, and placed in a sealed envelope to be used as a standard.

2.8.1.1.4 When supplied, a secondary standard shall be clearly marked as such and treated as a standard.

2.8.1.2 DNA Analysis

2.8.1.2.1 All extracts are considered work product. Any remaining extract shall be returned to the appropriate subitem for long term storage. Extraction controls shall also be retained.

2.8.1.2.2 When multiple samples have been retained from one item, the analyst can limit the number of samples per item to be extracted. A representative sample shall be tested initially. On an initial examination, 5 samples per piece of clothing or bedding should be sufficient to be extracted. Additional samples may be extracted as necessary. In sexual assault cases, where samples indicating penetration are available, additional samples are at analyst discretion. Samples where spermatozoa were detected and rated 1+ or above shall be extracted using the differential procedure. All samples AP+ or sperm positive may be extracted with a differential procedure at the analyst's discretion.

2.8.1.2.2.1 Acid phosphatase positive samples need not be extracted where there are extracted samples in which spermatozoa were present. Additional samples can be extracted at analyst's discretion.

2.8.1.2.2.2 If no spermatozoa were detected in a case and all samples retained for sexual assault only demonstrate the presence of acid phosphatase then a differential extraction procedure may be used at the analyst's discretion.

2.8.1.2.3 A quantification procedure shall be performed to estimate the quantity of extracted DNA.

2.8.1.2.4 If sufficient, a portion of the extracted DNA shall be amplified.

2.8.1.2.5 In order to be used in a comparison with evidentiary samples, any standard (primary or secondary) shall demonstrate a single source profile. Any exception to this rule shall only be made with the approval of the Technical Leader and documented in the case record.

2.8.1.2.6 A portion of the amplified DNA is removed and used in the typing method. The remaining amplified DNA, considered work product, shall be destroyed after the case has been administratively reviewed.

2.8.1.2.7 The amount of sample available for testing, the recoverability of DNA from the sample, and the quality of the DNA shall affect which tests are performed. The conclusions reached are based upon the analysis performed.

2.8.1.3 General Rules:

2.8.1.3.1 If one cutting has a quantity of DNA that would require concentration and another cutting from the same item has an appropriate quantity for analysis without additional treatment; it shall be left to the analyst's discretion as to whether or not both samples, or just the one sample will be typed.

2.8.1.3.2 Multiple reagent blanks (RB) may be extracted as part of a sample batch. All reagent blanks shall at a minimum be carried through quantification. Amplification shall be performed on the reagent blank demonstrating the highest quantification value. One reagent blank may be reserved without additional treatment to be used with those samples in the batch not requiring additional treatment. A batch is defined as all samples before a reagent blank(s) with no intervening reagent blanks.

2.8.1.3.3 Any additional treatment to a sample shall be done to the corresponding reagent blank. This would include but not be limited to additional Microcon® purifications, use of Centri-Sep columns, amplifying more than 5μl, or increased injection parameters. Reagent blanks shall not be diluted unless performed during the Biomek robotic processing of samples.

2.8.1.3.4 The extraction of question samples and known samples shall be performed separately (separated by time and/or space). This shall include a separate reagent blank for known and questioned samples and, when practical, use of separate extraction worksheets or separate columns of the same extraction worksheet.

2.8.1.3.5 Extraction and typing of background samples is often not beneficial, and is therefore not required, but may be done at the discretion of the DNA analyst to aid in the interpretation of case sample results. If a background sample is extracted it shall be typed if DNA was detected during the quantification

step. The background sample shall be concentrated only if the sample it is the background for is concentrated.

2.8.2 Crosslinker Procedure

Consumables (pipette tips, microtubes, columns, etc…) may be exposed in the crosslinker for 2 to 15 minutes before use in casework. Time in excess of 15 minutes shall have the approval of the Technical Leader documented in the case record.

The use of the crosslinker may reduce the presence of amplifiable DNA.

2.8.3 Microcon® Concentration Of Extracted DNA

2.8.3.1 Multiple Microcon® concentration steps may be performed to further clean up or concentrate any extract if desired. The same treatment shall be performed on the associated reagent blank (or a portion of) for each sample batch(es).

2.8.3.2 To a Microcon® 100 Concentrator add 100 µl TE^{-4}. Transfer the DNA containing supernatant to the concentrator.

2.8.3.3 Close the cap on the concentrator and spin in a microcentrifuge at 500 - 1200 rcf (relative centrifugal force) for at least 10 minutes.

2.8.3.4 Carefully remove the concentrator unit from the assembly and discard the fluid from the filtrate cup. Return the concentrator to the top of the filtrate cup. (Optionally 2.8.3.4 may be omitted.)

2.8.3.5 Remove the cap and add 200 µl nuclease free water (NFH2O) or TE^{-4} to the concentrator. Replace the cap and spin the assembly in a microcentrifuge at 500-1200 rcf for at least 10 minutes.

2.8.3.6 Remove the cap and add a measured volume of NFH_2O or TE^{-4} (40 µl is the recommended amount however, varied amounts can be added in some circumstances) to the concentrator. Remove the concentrator from the filtrate cup and carefully invert the concentrator into a new, labeled retentate cup. Discard the filtrate cup.

2.8.3.7 Spin the assembly in a microcentrifuge at 500-1200 rcf for at least 5 minutes.

2.8.3.8 Discard the concentrator. Cap the retentate cup.

2.8.3.9 Extracts can be stored refrigerated or frozen prior to quantification/amplification. Prior to use after storage the samples shall be vortexed briefly and spun in a microcentrifuge for about 5 seconds.

2.8.4 Centri-sep Clean-Up Of Extracts

2.8.4.1 Clean-up of extracts with Centri-sep columns may be performed at any time. The same treatment shall be performed on the associated reagent blank (or a portion of) for each sample batch.

2.8.4.2 Remove the top column cap and reconstitute the column by adding 800 µl of nuclease free water (NFH2O). Leave the column end stopper in place so the column can stand up by itself. Replace the column cap and hydrate the gel by shaking and inverting the column or vortexing briefly.

2.8.4.3 Allow the column to hydrate for at least 30 minutes at room temperature. Reconstituted columns may be refrigerated at 4° C overnight. Allow to warm up to room temperature before proceeding.

2.8.4.4 Remove the air bubbles from the column gel by inverting the column and sharply tapping the column, allowing the gel to slurry to the opposite end of the column. Stand the column up and allow the gel to settle while in a microtube rack.

2.8.4.5 First remove the column cap, and then remove the column end stopper from the bottom.

2.8.4.6 Allow the excess column fluid to drain into a wash tube (2 ml). If the fluid does not begin to flow immediately through the column apply gentle pressure to the top of the column to force the fluid to start through the column filter. This can be accomplished by snapping the cap on briefly. Discard this fluid.

2.8.4.7 Spin the column and wash tube at 750 rcf for 2 minutes. **NOTE: It is important to keep track of the position on the column using the orientation mark molded into the column.**

2.8.4.8 Optionally: discard eluate and rinse column with 150-200µl NFH_2O and spin for 2 minutes.

2.8.4.9 Blot any drop at the end of the column. Discard the wash tube. Do not allow the gel material to dry excessively.

2.8.4.10 Transfer the sample extract to the top of the column, without disturbing the gel surface. Keep track of amount of sample added to column (less than 50 µl is recommended).

2.8.4.11 Place the column into the sample collection tube (1.5 ml) and place both into the rotor. **Maintain proper column orientation.** The highest point of the gel media in the column should always point toward the outside of the rotor. Spin the column and the collection tube at 750 rcf for 2 minutes.

2.8.4.12 Discard the spin column and cap the sample collection tube.

Extracts can be stored refrigerated or frozen prior to quantification/amplification. Prior to use after storage, the samples shall be vortexed briefly and spun in a microcentrifuge for about 5 seconds.

2.8.5 Regular Organic Extraction With Microcon® Concentration Of All Sample Types Including Blood, Hair, Tissue, And Bone.

2.8.5.1 Place the sample in 1.5 ml microcentrifuge tube (See below for sampling protocols). Each group of samples being extracted shall include a reagent blank as the last sample in the batch.

2.8.5.2 To the sample add 300 µl of stain extraction buffer with DTT and 7.5 µl Proteinase K (Pro K) solution (10 mg/ml). Vortex for about 1 second and spin in a microcentrifuge for about 2 seconds or tap the tube to force the cutting into the extraction fluid. Analyst may increase the amounts of stain extraction buffer with DTT and Pro K proportionally for larger samples.

2.8.5.3 Incubate the tube at 56° C overnight (18 hours minimum/24 hours maximum). An extraction time of no less than 2 hours may be used for standards (blood or buccal swabs). A shortened extraction time may only be used with the approval of the Technical Leader (documented in case record) for any other sample types.

2.8.5.4 Spin in a microcentrifuge for about 2 seconds to force the condensate into the bottom of the tube. (For hairs, bones, or tissue it may be preferable to transfer supernatant to a clean microcentrifuge tube before proceeding to 2.8.5.7).

2.8.5.5 Using a wooden applicator stick, remove the cutting and proceed to 2.8.5.7 or

2.8.5.6 Transfer the cutting into a Spin-X basket insert. Place the basket insert into the tube containing the stain extract. Spin in a microcentrifuge for about 5 minutes. Remove and discard the basket insert.

2.8.5.7 In a fume hood, add 300 µl of phenol/chloroform/isoamyl alcohol (p/c/i) to the stain extract and vortex the mixture to attain a milky emulsion. Spin in a microcentrifuge until completely separated (for at least 3 minutes) at 14,000 rcf. Multiple phenol/chloroform/isoamyl alcohol extractions may be performed to further clean up an extract if desired. If larger volumes are used in step 2.8.5.2, equivalent volumes of p/c/i shall be used. The same treatment shall be performed to any associated reagent blanks.

2.8.5.8 To a Microcon® 100 Concentrator add 100 µl TE^{-4}. Transfer the aqueous phase (top layer) from the tube in 2.8.5.7 to the concentrator in the fume hood. Avoid pipetting organic solvent from the tube into the concentrator.

2.8.5.9 Close the cap on the concentrator and spin in a microcentrifuge at 500-1200 rcf for at least 10 minutes.

2.8.5.10 Remove the cap and add 200 µl NFH_2O or TE^{-4} to the concentrator. Replace the cap and spin the assembly in a microcentrifuge at 500-1200 rcf for at least 10 minutes. (If necessary the fluid from the filtrate cup may be discarded prior to adding the 200 µl rinse).

2.8.5.11 Remove the cap and add a measured volume of NFH_2O or TE^{-4} (40 µl is the recommended amount however, varied amounts can be added in some circumstances) to the concentrator. If desired, the sample can be

brought up with additional TE^{-4} or water by adding it to the retentate cup. Remove the concentrator from the filtrate cup and carefully invert the concentrator onto a new, labeled retentate cup. Discard the filtrate cup.

2.8.5.12 Spin the assembly in a microcentrifuge at 500-1200 rcf for at least 5 minutes.

2.8.5.13 Discard the concentrator. Cap the retentate cup.

2.8.5.14 Extracts can be stored refrigerated or frozen prior to quantification/amplification. Prior to use after storage the samples shall be vortexed briefly and spun in a microcentrifuge for about 5 seconds.

2.8.5.15 Sampling Protocols

2.8.5.15.1 Sample size may vary from indicated values depending on many factors including but not limited to total sample available and serology test results.

2.8.5.15.2 The analyst shall consume only as much of the available sample as is needed. Normally not more than half of the available sample will be used for testing. If it is necessary to consume an entire sample in testing or an amount such that the remaining portion is insufficient for additional testing, the analyst shall first receive permission from the prosecutor or investigator and document such in the case record. This may be done at the time of submission.

2.8.5.15.3 **Blood**: Sample approximately 3 X 3 mm from a blood stain or 3 µl of whole blood.

2.8.5.15.4 **Envelope**: Sample approximately 1 X 1 cm of a gummed envelope flap or stamp. Cut sample into smaller pieces before placing in extraction tube. Alternatively, may swab gummed area or wash with three 50 µl rinses and place each rinse into the extraction tube.

2.8.5.15.5 **Cigarette**: Sample ½ to all of paper approximately 5 mm down from end or ½ of the paper and filter approximately 5 mm down (Filter not to be used when extracting with Maxwell Robot). Cut sample into smaller pieces before placing in extraction tube.

2.8.5.15.6 **Epithelial Swabs**: Sample from 1/3 to entire swab used to collect epithelial cells. May extract two swabs in two separate extraction tubes, combining at step 2.8.5.8 above. The volume of the associated RB(s) should also be doubled.

2.8.5.15.7 **Hair:** Rinse root end of hair in 100% ethanol and then dH2O before sampling 1 cm of root end. If desired a second 1 cm of hair may be extracted separately as a sample blank.

2.8.5.15.8 **Bone (See 2.8.9 for more detailed instructions)**

2.8.5.15.8.1 Remove soft tissue adhering to bone sample.

2.8.5.15.8.2 Immerse and agitate at least 1 cubic inch of bone in cold distilled water. If necessary, the specimen can be further

cleaned by immersion in ethanol and ethyl ether for about 15 minutes each.

2.8.5.15.8.3 Allow the sample to dry.

2.8.5.15.8.4 Crush bones into a fine powder. If available, samples may be immersed in liquid nitrogen for about 15-30 seconds to freeze. Bone samples may require multiple freezing/crushing steps.

2.8.5.15.8.5 Larger samples than indicated may be used, as needed, with the amounts of stain extraction buffer with DTT and Pro K increased proportionally.

2.8.5.15.9 **Tissue**

2.8.5.15.9.1 Cut a piece of tissue approximately 3 X 3 mm.

2.8.5.15.9.2 If tissue is immersed in liquid nitrogen, it shall also be crushed into a fine powder. Transfer powder or (if not crushed) tissue sample to a microcentrifuge tube.

2.8.5.15.9.3 Larger samples than indicated may be used, as needed, with the amounts of stain extraction buffer with DTT and Pro K increased proportionally.

2.8.6 Organic Extraction With Microcon® Concentration Of Semen Stains

2.8.6.1 Place the sample in 1.5 ml microcentrifuge tube (See below for sampling protocols). Each group of samples being extracted shall include reagent blanks (sperm and non-sperm fraction) as the last samples in the batch.

2.8.6.2 To the sample add:

400 µl Tris/EDTA/NaCl

25 µl 20% Sarkosyl

75 µl NFH_2O

5 µl Pro K (10 mg/ml)

2.8.6.3 Vortex for about 1 second and spin in a microcentrifuge for about 2 seconds to force the material into the extraction fluid.

2.8.6.4 Incubate at 37°C for 2 hours.

2.8.6.5 Spin in a microcentrifuge for about 2 seconds to force the condensate into the bottom of the tube.

2.8.6.6 Using a wooden applicator stick, remove the cutting and spin in a microcentrifuge for about 5 minutes. Alternately transfer the cutting into a Spin-X basket insert. Place the basket insert into the tube containing the stain extract. Spin in a microcentrifuge for about 5 minutes. Remove and discard the basket insert.

2.8.6.7 While being very careful to not disturb any pelleted material, remove the supernatant fluid from the extract and place it into a new, labeled tube. This supernatant is the non-sperm cell fraction. Analysis of the non-sperm cell fraction resumes at 2.8.6.14. The pellet remaining in the tube is the sperm cell pellet.

2.8.6.8 Wash the sperm cell pellet by resuspending it in 500 µl digest/wash buffer, vortexing the suspension briefly, and spinning the tube in a microcentrifuge at 10,000 to 15,000 rcf for about 5 minutes. Remove and discard the supernatant fluid, being careful not to disturb the cell pellet.

2.8.6.9 Repeat 2.8.6.8 four additional times for a total of five washes of the cell pellet. Approval, documented in the case record, from the Technical Leader is required to use less than the five washes.

2.8.6.10 To the tube containing the washed pellet add:

150 µl Tris/EDTA/NaCl

50 µl 20% Sarkosyl

150 µl NFH_2O

10 µl Pro K (10 mg/ml)

40 µl 0.39M DTT

2.8.6.11 Close the tube caps and vortex for about 1 second and spin in a microcentrifuge for 2 seconds to force all fluid and material to the bottoms of the tubes.

2.8.6.12 Incubate at 37°C for 2 hours.

2.8.6.13 Spin in a microcentrifuge for about 2 seconds to force the condensate into the bottom of the tube.

2.8.6.14 To the tube containing the sperm cell pellet and to the tube containing the non sperm fraction, add 400 µl phenol/chloroform/isoamyl alcohol in the fume hood. Vortex the mixture briefly at low speed (in the fume hood) to attain a milky emulsion. Spin in a microcentrifuge until completely separated (for at least 5 minutes) at ~14,000 rcf. Multiple phenol/chloroform/isoamyl alcohol extractions may be performed to further clean up an extract if desired. The same treatment shall be performed to any associated reagent blanks.

2.8.6.15 To a Microcon® 100 Concentrator add 100 µl TE^{-4}. Transfer the aqueous phase (top layer) from the tube in 2.8.6.14 to the concentrator in the fume hood. Avoid pipetting organic solvent from the tube into the concentrator.

2.8.6.16 Close the cap on the concentrator and spin in a microcentrifuge at 500-1200 rcf for at least 10 minutes.

2.8.6.17 Carefully remove the concentrator unit from the assembly and discard the fluid from the filtrate cup. Return the concentrator to the top of the filtrate cup.

2.8.6.18 Remove the cap and add 200 µl NFH_2O or TE^{-4} to the concentrator. Replace the cap and spin the assembly in a microcentrifuge at 500-1200 rcf for at least 10 minutes.

2.8.6.19 Remove the cap and add a measured volume of NFH_2O or TE^{-4} (40 µl is the recommended amount however, varied amounts can be added in some circumstances) to the concentrator. If desired, the sample can be brought up with additional water by adding it to the retentate cup. Remove the concentrator from the filtrate cup and carefully invert the concentrator onto a new, labeled retentate cup. Discard the filtrate cup.

2.8.6.20 Spin the assembly in a microcentrifuge at 500-1200 rcf for at least 5 minutes.

2.8.6.21 Discard the concentrator. Cap the retentate cup.

2.8.6.22 Extracts can be stored refrigerated or frozen prior to quantification/amplification. Prior to use after storage the samples shall be vortexed briefly and spun in a microcentrifuge for about 5 seconds.

2.8.6.23 Sampling Protocols

2.8.6.23.1 Sample size may vary from indicated values depending on many factors including but not limited to total sample available and serology test results.

2.8.6.23.2 The analyst shall consume only as much of the available sample as is needed. Normally not more than half of the available sample will be used for testing. If it is necessary to consume an entire sample in testing or an amount such that the remaining portion is insufficient for additional testing, the analyst shall first receive permission from the prosecutor or investigator and document such in the case record. This may be done at the time of submission.

2.8.6.23.3 **Swabs**: Sample from ¼ to an entire swab. If multiple swabs have been collected together, analysts may sample a portion of each swab and combine into one extraction tube.

2.8.6.23.4 **Clothing**: Sample approximately a 5 X 5 mm cutting.

2.8.6.23.5 **Smear Slides**: Use a moistened swab and swab entire smear, including underside of coverslip. Sample swab as above.

2.8.6.23.6 **Vaginal Wash**: Spin down vaginal wash and collect pellet on one or more swabs. Sample swab(s) as above. Alternatively may pipette ~ ½ of pellet (not to exceed 50 µl) into 1.5 ml extraction tube prior to collecting remaining pellet on swab(s).

2.8.7 Maxwell®16 Protocol For Blood, Hair, Saliva, Epithelial Cell Samples And Blood Or Buccal Swab Standards.

2.8.7.1 Preparation of Sample: (see sampling protocols with Organic extraction)

2.8.7.1.1 Prepare Buffer/Pro K/DTT Master Mix

2.8.7.1.1.1 For each sample, combine 350 µl Incubation Buffer with 10 µl Pro K (18 mg/ml) and 40 µl DTT (1M). Alternatively, Stain Extraction Buffer for Automation (SEBA) may be substituted for Incubation Buffer. *Example: for 16 samples, combine 5600 µl Incubation Buffer (or SEBA) with 160 µl Pro K and 640 µl DTT.*

2.8.7.1.1.2 Place sample at the bottom of a labeled 1.5 ml microcentrifuge tube and add 400 µl Buffer/Pro K/DTT Master Mix. Close the tube cap, vortex briefly and spin down. Each group of samples being extracted shall include a reagent blank as the last sample in the batch.

2.8.7.1.2 Incubate the sample at 56°C for 1 hour.

2.8.7.1.3 Vortex samples briefly and spin down.

2.8.7.1.4 Add 200 µl of Lysis Buffer to each sample.

2.8.7.1.5 Vortex sample briefly and spin down.

2.8.7.1.6 Remove cutting and proceed to 2.8.7.1.8 or:

2.8.7.1.7 Place the cutting into a spin basket in the labeled 1.5 ml microcentrifuge tube. *Optionally the Lysis Buffer may also be transferred to the spin basket.* Close the microtube cap. Centrifuge for at least 2 minutes at maximum speed. Carefully remove and discard the spin basket.

2.8.7.1.8 Close the lid of the microtube and save until ready for automated DNA extraction. Do not refrigerate or freeze sample. Leave processed sample at room temperature (~22-25°C) overnight, if necessary.

2.8.7.2 Maxwell® 16 Instrument Automated DNA Purification

2.8.7.2.1 Place the number of cartridges to be used into the cartridge preparation rack. Each cartridge corresponds to one sample. Place each cartridge into the rack with barcode facing away from the numbered side of the rack. Hold the cartridge firmly and remove the seal.

2.8.7.2.2 Place one plunger into well #8 of each cartridge.

2.8.7.2.3 Transfer the entire sample into well #1.

2.8.7.2.4 Place 0.5 ml elution tubes (properly labeled) into the elution tube slots in rack. Add 50 µl of Elution Buffer.

2.8.7.2.5 Turn on the Maxwell® 16 Instrument. Verify LEV and FNSC modes are displayed.

2.8.7.2.6 Scroll to “Run” on the Menu screen and press the “Run/Stop” button.

2.8.7.2.7 Open the door when prompted to do so on the LCD display. Press the “Run/Stop” button to extend the platform.

2.8.7.2.8 Place the cartridge rack into the Maxwell® 16 Instrument.

2.8.7.2.9 Press the “Run/Stop” button. The platform will retract. Close the door.

2.8.7.2.10 Upon method completion, open the instrument door. The plungers should be located in Well #8 at the end of the run. Check to make sure that all of the plungers have been removed from the magnetic rod assembly. If the plungers have not been removed, push them down gently by hand and remove them from the magnetic rod assembly.

2.8.7.2.11 Press the “Run/Stop” button to extend the platform.

2.8.7.2.12 Remove the Elution Tubes from the heated elution tube slots, as soon as possible and close the top on each tube. (Leaving the heated elution tubes in the instrument for an extended period of time can result in evaporation of the sample.) Samples can be stored at 4°C or frozen. Prior to use after storage, the samples shall be vortexed briefly and spun in a microcentrifuge for about 5 seconds.

2.8.7.2.13 Remove cartridges and plungers from the instrument platform and discard.

2.8.7.2.14 When completed, clean the Maxwell® 16 Instrument and cartridge rack with 70% ethanol and turn off machine.

2.8.8 Maxwell®16 Protocol For Differential Extractions.

2.8.8.1 Preparation of Samples: (see sampling protocols in 2.8.6.23)

2.8.8.1.1 Prepare Master Mix

2.8.8.1.1.1 For each sample, combine 400 µl Tris/EDTA/NaCl, 10 µl 20% Sarkosyl, 90 µl NFH_2O and 5 µl Proteinase K (10 mg/ml).

2.8.8.1.1.2 Place sample at the bottom of a labeled 1.5 ml microcentrifuge tube and add 505 µl of the Master Mix. Close the tube cap, vortex briefly and spin down. Each group of samples being extracted shall include a reagent control blank as the last sample in the run.

2.8.8.1.2 Incubate the sample at 37°C for 2 hours.

2.8.8.1.3 Vortex samples briefly and spin down.

2.8.8.1.4 Remove cutting and place into spin basket. Close the cap and centrifuge at room temperature for at least 2 minutes at maximum speed. Carefully remove and discard the spin basket.

2.8.8.1.5 Carefully remove the supernatant to a clean, labeled micro-centrifuge tube. This is the non-sperm fraction.

2.8.8.1.6 Wash the sperm pellet with 500 µl of Digest Wash Buffer and spin down for about 5 minutes. Carefully remove and discard the supernatant.

2.8.8.1.7 Repeat step 2.8.8.1.6 four times for a total of five washes. Approval, documented in the case record, from the Technical Leader is required to use less than the five washes.

2.8.8.1.8 Lysis buffer step

2.8.8.1.8.1 For each sample pair (sperm and non-sperm fraction) combine 600 µl of Lysis Buffer with 6 µl of 1M DTT.

2.8.8.1.8.2 Add 400 µl of the Lysis Buffer master mix to each sperm cell fraction sample and 200 µl to each non-sperm fraction sample.

2.8.8.1.9 Vortex sample briefly and spin down.

2.8.8.1.10 Save until ready for automated DNA extraction. Do not refrigerate or freeze sample. Leave processed sample at room temperature (~22-25°C) overnight, if necessary.

2.8.8.2 Maxwell® 16 Instrument Automated DNA Purification

2.8.8.2.1 Place the number of cartridges to be used into the cartridge preparation rack. Each cartridge corresponds to one sample. Place each cartridge into the rack with the tube holder facing towards the numbered side of the rack. Hold the cartridge firmly and remove the seal.

2.8.8.2.2 Place one plunger into well #8 of each cartridge.

2.8.8.2.3 Transfer the entire sample into well #1.

2.8.8.2.4 Place 0.5 ml elution tubes (properly labeled) into the elution tube holder. Add 50 µl of Elution Buffer.

2.8.8.2.5 Turn on the Maxwell® 16 Instrument. Verify LEV and FNSC modes are displayed.

2.8.8.2.6 Scroll to “Run” on the Menu screen and press the “Run/Stop” button.

2.8.8.2.7 Open the door when prompted to do so on the LCD display. Press the “Run/Stop” button to extend the platform.

2.8.8.2.8 Place the cartridge rack into the Maxwell® 16 Instrument.

2.8.8.2.9 Press the “Run/Stop” button. The platform will retract. Close the door.

2.8.8.2.10 Upon method completion, open the instrument door. The plungers should be located in Well #8 at the end of the run. Check to make sure that all of the plungers have been removed from the magnetic rod assembly. If the plungers have not been removed, push them down gently by hand and remove them from the magnetic rod assembly.

2.8.8.2.11 Press the “Run/Stop” button to extend the platform.

2.8.8.2.12 Remove the Elution Tubes from the heated elution tube slots, as soon as possible and close the top on each tube. (Leaving the heated elution tubes in the instrument for an extended period of time can result in evaporation of the sample.) Samples can be stored at 4°C or frozen. Prior to use after storage, the samples shall be vortexed briefly and spun in a centrifuge for about 5 seconds.

2.8.8.2.13 Remove cartridges and plungers from the instrument platform and discard.

2.8.8.2.14 When completed, clean the Maxwell® 16 Instrument and cartridge rack with 70% ethanol and turn off machine.

2.8.9 SAMPLING PROTOCOL FOR BONE AND TEETH

2.8.9.1 Sample cleaning/preparation methods (e.g., scraping, heating, etc.) that best address the variability of sample quality may be used as necessary.

2.8.9.2 Remove and separate any soft tissue adhering to sample with a sterilized scalpel.

2.8.9.3 Immerse and wash sample in distilled water or a 1:10 bleach solution. A small brush or toothbrush may be used to gently remove any dirt/debris from the outer surfaces and exposed inner surfaces of sample.

2.8.9.3.1 Small bone samples with difficult-to-reach surfaces may be cleaned by sealing the sample in a 50 ml conical tube containing distilled water or a 1:10 bleach solution, then vortexing briefly, changing out the solution, and repeating as necessary.

2.8.9.4 Rinse sample in distilled water and allow sample to dry.

2.8.9.5 Pulverize sample by crushing or by use of a freezer/mill. Larger samples (e.g., bones and adult teeth) may need to be initially reduced in size to accommodate the method of pulverization. This may be accomplished by use of a hammer, handsaw, or Dremel® tool. Note: any medical/dental /restorative work on a bone or tooth sample shall be removed prior to pulverization.

2.8.9.5.1 Mechanical Crushing

2.8.9.5.1.1 Mechanical Crushing

2.8.9.5.1.1.1 Larger samples may initially need to be reduced to ~0.5 in. fragments or smaller to accommodate the physical size of the crushing tool.

2.8.9.5.1.1.2 Crush bone sample using crushing tool and mallet. Optionally, sample may be immersed in liquid nitrogen for ~1–10 minutes to aid in crushing.

2.8.9.5.1.2 SPEX SamplePrep® 6770 Freezer/Mill

2.8.9.5.1.2.1 Sample must initially be reduced to ~0.25 in. fragments (width of a pencil eraser) or smaller to accommodate the requirements of the mill. Larger fragments will decrease pulverization efficiency and may damage the mill.

2.8.9.5.1.2.2 Insert a sterilized blunt end cap into a sterilized grinding cylinder.

2.8.9.5.1.2.3 Add the sample to the cylinder. Do not add sample in excess of 1/3 total volume of the cylinder.

2.8.9.5.1.2.4 Insert a steel impactor into the cylinder and seal the cylinder with a sterilized flanged end cap.

2.8.9.5.1.2.5 Slowly add liquid nitrogen to the freezer/mill until it reaches the fill mark. An initial amount of liquid nitrogen

will quickly boil off as the tank cools. When the liquid settles, add additional liquid nitrogen up to the fill mark. Initial cooling of the instrument will consume ~4–5 L of liquid nitrogen, and each sample pulverization will require an additional ~1 L liquid nitrogen.

2.8.9.5.1.2.5.1 Always handle liquid nitrogen containers or any item exposed to liquid nitrogen (including grinding cylinders) with cryogenic gloves.

2.8.9.5.1.2.6 Load assembled sample vial into the freezer/mill chamber (blunt end first). Optionally, up to two additional samples may also be prepared in vials and placed in the pre-cooling chamber above the freezer/mill chamber.

2.8.9.5.1.2.7 Close the freezer/mill and pulverize the sample using the following protocol: Cycles: 4, Pre-cooling: 10 min, Run: 2 min, Cool: 2 min, CPS (cycles per second): 10. Regularly check the control panel on the freezer/mill to ensure that sufficient liquid nitrogen is remaining in the tank. If “LOW LN LEVEL” is displayed, pause the protocol by pressing “PAUSE”, open the freezer/mill, and add liquid nitrogen to the fill mark, then close the freezer/mill and resume the protocol.

2.8.9.5.1.2.8 When the program is complete, open freezer/mill and remove vial from freezer/mill chamber. To conserve liquid nitrogen, any subsequent samples should be immediately loaded into the mill and pulverized as described above. If additional samples were loaded in the pre-cooling chamber during the initial sample run, they may be pulverized with the following protocol (omitting the pre-cooling phase): Cycles: 4, Pre-cooling: 0 min, Run: 2 min, Cool: 2 min, CPS: 10.

2.8.9.5.1.2.9 Allow vial to warm up for ~5–10 minutes before using the cap extractor tool to open the vial. If the sample was “sticky” or “tacky” prior to pulverization, it may be necessary to open the vial earlier to aid removal. Transfer the contents of the vial into a sterilized weigh boat and separate the impactor from the sample. A sterilized spatula may be used to aid sample removal.

2.8.9.5.1.3 Transfer the pulverized sample into one or more microcentrifuge tubes. Mechanical crushing typically generates small sample fragments accompanied by some powder, while a freezer/mill typically converts an entire sample to a very fine powder. Typically, 1 adult tooth with root (or similarly sized bone sample), or 1 g of bone, will generate fragments/powder to fill two 1.5 ml microcentrifuge tubes about halfway full, leaving sufficient space for extraction reagents.

2.8.9.5.1.4 Larger samples than indicated may be used, as needed, with the amounts of stain extraction buffer with DTT and Pro K increased proportionally.

2.8.10 Real-Time PCR Quantification Using Quantifiler® Duo

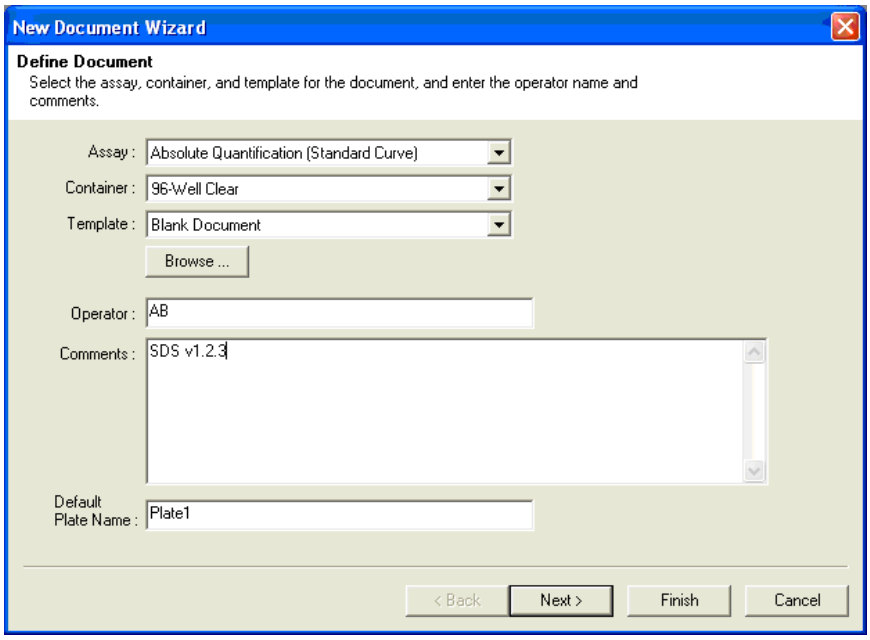
2.8.10.1 Create a Plate Document (Note: The plate document shall be created and saved on the computer prior to running the quantification plate on the instrument.)

2.8.10.1.1 Turn on the computer.

2.8.10.1.2 Turn on the Real-Time PCR instrument (must be on prior to opening the software).

2.8.10.1.3 Open the Applied Biosystems 7500 System Sequence Detection Software v 1.2.3.

2.8.10.1.4 Set up the Plate Document as described below: File ⇒New



Assay: **Absolute Quantification (Standard Curve)**
Container: **96-Well Clear**
Template: Quantifiler® Duo
Operator: your name
Plate Name: ”First lab file on plate_date_plate#” ex. “00A1234_01Jan11_01”

Click **Finish**.

2.8.10.1.5 Sample names and case numbers can be entered into each well at this time or it can be done after the run has completed. Each sample shall be uniquely identified.

2.8.10.1.6 Save the plate document at this time.

2.8.10.1.7 Leave the plate document open.

2.8.10.2 Preparation Of The Quantification Plate

2.8.10.2.1 The Quantifiler® Duo kit should be stored at -15 to -25 °C upon receipt. Once thawed, all components are to be stored at 2 to 8 °C.

2.8.10.2.2 The Quantifiler® Duo PCR Primer Mix and the Quantifiler® Duo PCR Reaction Mix shall be protected from excessive exposure to light.

2.8.10.2.3 The preparation of the standard curve and the plate shall be performed in the PCR amplification set-up area.

2.8.10.2.4 The standard curve is stable for up to 2 weeks at 2 to 8°C when prepared using Quantifiler® Duo Dilution Buffer.

2.8.10.2.5 **Prepare the Standard Curve**

2.8.10.2.5.1 Allow the entire Quantifiler® Duo kit to thaw prior to first use.

2.8.10.2.5.2 Label eight microcentrifuge tubes Std. 1 through Std. 8.

2.8.10.2.5.3 Dispense the required amount of Quantifiler® Duo dilution buffer to each tube. (See table below for examples)

2.8.10.2.5.4 Vortex the Quantifiler® Duo DNA Standard [200 ng/µl stock] for 3 to 5 seconds.

2.8.10.2.5.5 Add the calculated amount of Quantifiler® Duo DNA Standard to Std. 1 and mix thoroughly.

2.8.10.2.5.6 Using a new pipette tip, add the calculated amount of Std. 1 to Std. 2 and mix thoroughly.

2.8.10.2.5.7 Using a new pipette tip each time, continue diluting each successive concentration until the dilution series is complete.

Standard Curve	Dilution Series Minimum Amount	Dilution Series Example Amount	Dilution Factor
Std. 1 [50.0 ng/µl]	30 µl dilution buffer + 10 µl of 200 ng/µl DNA stock	90 µl dilution buffer + 30 µl of 200 ng/µl DNA stock	4X
Std. 2 [16.7 ng/µl]	20 µl dilution buffer + 10 µl Std. 1	60 µl dilution buffer + 30 µl Std. 1	3X
Std. 3 [5.56 ng/µl]	20 µl dilution buffer + 10 µl Std. 2	60 µl dilution buffer + 30 µl Std. 2	3X
Std. 4 [1.85 ng/µl]	20 µl dilution buffer + 10 µl Std. 3	60 µl dilution buffer + 30 µl Std. 3	3X
Std. 5 [0.620 ng/µl]	20 µl dilution buffer + 10 µl Std. 4	60 µl dilution buffer + 30 µl Std. 4	3X
Std. 6 [0.210 ng/µl]	20 µl dilution buffer + 10 µl Std. 5	60 µl dilution buffer + 30 µl Std. 5	3X
Std. 7 [0.068 ng/µl]	20 µl dilution buffer + 10 µl Std. 6	60 µl dilution buffer + 30 µl Std. 6	3X
Std. 8 [0.023 ng/µl]	20 µl dilution buffer + 10 µl Std. 7	60 µl dilution buffer + 30 µl std. 7	3X

2.8.10.3 Prepare the Reactions

2.8.10.3.1 Each plate shall contain a standard curve series run in duplicate and a Non-Template Control (NTC).

2.8.10.3.2 Determine the number of samples to be quantified, including standards and the non-template control. Add 3 to 5 reactions to this number to compensate for the loss that occurs during reagent transfers.

2.8.10.3.3 Using the Master Mix worksheet, calculate the required amount of each component of the master mix. Multiply the volume per reaction (µl) by the total number of reactions.

PCR Master Mix Component	Volume per Reaction (µl)
Quantifiler® Duo Primer Mix	10.5
Quantifiler® Duo PCR Reaction Mix	12.5
Total Volume	23.0

2.8.10.3.4 Vortex the Primer Mix 3 to 5 seconds, then centrifuge briefly.

2.8.10.3.5 Swirl the PCR Reaction Mix gently before using. **Do not vortex**.

2.8.10.3.6 Pipette the required volumes of each component into a microcentrifuge tube.

2.8.10.3.7 Vortex the Master Mix 3 to 5 seconds, then centrifuge briefly.

2.8.10.3.8 Dispense 23 µl of the Master Mix into each reaction well of a 96-well optical plate.

2.8.10.3.9 Add 2 µl of sample, standard, or non-template control (dilution buffer) to the appropriate wells.

2.8.10.3.10 Seal the reaction plate with an Optical Adhesive Cover. Run the edge of the cover applicator between the rows and columns of the wells to ensure that all wells are sealed properly.

2.8.10.3.11 Spin the plate using either a centrifuge or a salad spinner to remove any bubbles and force samples into the bottom of each well.

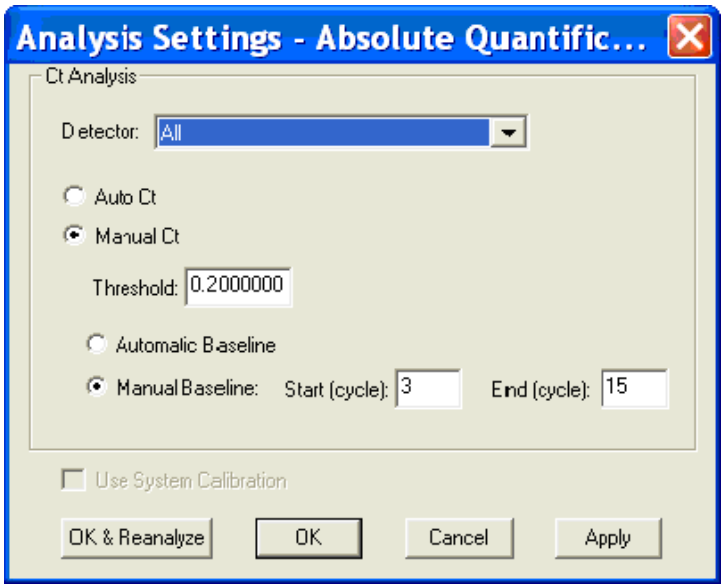
2.8.10.3.12 Place the plate in the instrument.

2.8.10.3.13 Select the **Instrument** tab of the plate document and click “Start”.

2.8.10.3.14 When the run is finished, click “Okay”.

2.8.10.4 Analyze The Plate

2.8.10.4.1 Verify the analysis settings as displayed below: **Analysis ⇒ Analysis Settings.**



2.8.10.4.2 Analyze the plate by clicking the Green Arrow (►) on the toolbar or select **Analysis ⇒ Analyze.**

2.8.10.4.3 Select the **Results** tab

2.8.10.4.3.1 At this point, there are several sub-tabs to view/verify data **(Standard Curve; Amplification Plot; Report;** etc.).

2.8.10.4.3.2 To view results for all wells: select all wells of the plate document by clicking on the small box left of column 1 and above row A.

2.8.10.4.3.3 If you make any changes after analyzing the plate (i.e. omit a well; change a standard to unknown), you must re-analyze the plate again as described in 2.8.10.4.2.

2.8.10.4.4 Examine the Human and Male standard curves. (See Interpretation Guidelines for Real-time PCR Quantification for additional evaluation of the standard curves.)

2.8.10.4.4.1 Slope: Acceptable range -3.0 to -3.6; average -3.3.

2.8.10.4.4.2 Y-intercept: Acceptable between 28 and 31 (may be < 28 on some instruments).

2.8.10.4.4.3 R^2: shall be ≥ 0.98

2.8.10.4.5 Check for inhibition of samples by looking at the C_T values for the IPC.

2.8.10.4.6 Save data under each associated laboratory case number and request folder located in the analysts' folders on the server.

2.8.10.4.7 At a minimum the required documents for the case record shall be generated. The required documents include the plate document displaying quantification values, the plate document displaying C_T values, the Duo Human standard curve graph, and the Duo Male standard curve graph if the evidence is from a sexual assault or if the amount of male DNA will be used for any sample evaluation.

2.8.10.5 Interpretation Guidelines For Real-Time PCR Quantification

2.8.10.5.1 **Controls**

2.8.10.5.1.1 **Standard Curve**: The purpose of the standard curve is to evaluate the quality of the results from the quantification standard reactions.

2.8.10.5.1.1.1 **Slope:** indicates the PCR amplification efficiency for the assay. A slope of -3.3 indicates 100% amplification efficiency. Scientific rounding rules apply. The analyst shall obtain documented Technical Leader approval to use data when the slope is outside the accepted range.

Kit	Range	Average
Quantifiler® Duo	-3.0 to -3.6	-3.3

2.8.10.5.1.1.2 **Y-intercept:** indicates the expected C_T value for a sample with Quantity = 1 (i.e. 1 ng/µl). If the value is < 28, the target amount of DNA for amplification may need to be increased. The analyst shall obtain documented Technical Leader approval to use data when the Y-intercept value is <28.

2.8.10.5.1.1.3 **R^2 value:** a measure of the closeness of fit between the standard curve regression line and the individual C_T data points of the quantification standard reactions. A value of

1.00 indicates a perfect fit between the regression line and the data points. This value shall be ≥ 0.980. If the R2 value is <0.98 the test is inconclusive and should be repeated. The Technical Leader shall be notified with documentation in the case record.

2.8.10.5.1.2 **Internal PCR Control (IPC):** The purpose of the IPC is to distinguish between a true negative sample result and reactions affected by the presence of PCR inhibitors, assay setup, and a chemistry or instrument failure.

Duo Human (VIC) and/or Duo Male (FAM)	IPC (NED)	Interpretation	Suggestions / Options
No amplification	Amplification	True negative (No human DNA detected)	No further analysis of sample
No amplification	No amplification	Invalid result	Re-Microcon® and/or Centri-sep; Re-extract; or dilute and re-quantify
Amplification (low C_T)	No amplification or High C_T	Inconclusive IPC result	Make dilutions and re-quantify (optional)
Amplification (high C_T)	No amplification or High C_T	Partial PCR Inhibition	Re-Microcon® and/or Centri-sep; Re-extract; or dilute and re-quantify

2.8.10.5.1.2.1 A low C_T value of < 20 for VIC is an indication of competition between the human-specific and IPC PCR reactions due to very high concentrations of the human template DNA. An analyst should make dilutions of the sample and run on another real-time PCR plate.

2.8.10.5.1.2.2 A high C_T value of >30 for the IPC (NED) is an indication of an inhibitor in the sample. An analyst may choose to re-Microcon® and/or centri-sep the sample prior to amplification and typing of the sample. The C_T value for the IPC should range from 20 to 30, with an average of around 27.

2.8.10.5.1.2.3 A profile can sometimes be obtained from a sample in which the IPC is undetected. It is at the discretion of the analyst whether or not to continue processing a sample in which the IPC is undetected. If the sample is not carried throughout the testing process, it shall be reported out as inconclusive.

2.8.10.5.1.3 **Non-Template Control (NTC):** Contains PCR reagent, but no template DNA. Occasionally, a value may be given for the NTC due to background fluorescence. The analyst may proceed with amplification and typing of the samples using caution with interpretation of any profiles obtained from the samples.

2.8.10.5.1.4 Occasionally there will be an outlier on the amplification plot of the standard curve. Up to 2 points may be omitted from the Human curve by changing the task assignment for VIC from “standard” to “unknown” in the Well Inspector. (Or “omit well” if point is to be dropped from both standard curves.) Be sure to reanalyze the plate to incorporate the changes. The analyst shall need documented supervisor approval to omit additional points in the standard curve. The Technical Leader shall be notified with documentation in the case record.

2.8.10.5.1.5 At the lowest concentration point of the Male standard curve, there are only ~3 to 4 copies of the haploid target locus per µl. Because of stochastic effects when using the lowest concentration point, the C_T values are more variable and may affect the closeness of fit (R^2 value) between the standard curve regression line and the individual points of the standard curve. For this reason, one or both std. 8 points may be omitted from analysis. Up to 2 additional points may be omitted from the Male standard curve. Be sure to reanalyze the plate to incorporate the changes. The analyst shall need documented supervisor approval to omit additional points in the standard curve. The Technical Leader shall be notified with documentation in the case record.

2.8.10.5.1.6 If the ratio of female to male DNA in a single sample exceeds 25:1 in instances where it is necessary to differentiate male and female profiles, no autosomal STR analysis of that sample shall be performed and Y-STR analysis is recommended. Non-sperm cell fractions where the sperm cell fraction is being analyzed shall be amplified regardless of the amount of male DNA present. If the ratio of female to male DNA in a single sample exceeds 10:1, the relative value should be weighed. Y-STR or other analysis may be considered in this situation. If sufficient sample is available, autosomal STR analysis may be attempted. Note: the female DNA contribution is determined by subtracting the quantity of male DNA from the quantity of human DNA. (human-male:male).

2.8.10.5.1.7 Dynamic range: If the quantity of Human DNA detected in the sample is below 0.01ng/µl no additional autosomal STR analysis of that sample shall be performed. When the quantity of Human DNA detected is between 0.023 and 0.01ng/µl the sample need not be amplified if usable results are obtained from other samples.

2.8.10.5.1.8 Dynamic range: The analyst should make a reasonable effort to target an amount of DNA during extraction not to exceed 50 ng/µl. If the sample is > 50 ng/µl, dilutions shall be made and re-quantified if they are to be amplified.

2.8.10.6 Quantification values from the Human target (VIC) shall be used for amplification of STR's.

2.8.10.7 Because the quantification values may have many significant figures, the analyst may truncate the value to one digit past the decimal point, but only after the value has been multiplied by 5 for amplification. (i.e. 1A = 1.59 ng/µl x 5 µl = 7.95 ng/5 µl = 7.9).

2.8.11 Powerplex® Hot Start (HS) 16 Introduction (Applied Biosystems 3130 Genetic Analyzer)

2.8.11.1 The Promega PowerPlex® 16 HS System allows for the amplification of fifteen short tandem repeat (STR) loci and the Amelogenin locus found on the X and Y chromosome (see chart on next page). The amplification occurs in a single reaction tube and detection occurs by a single capillary electrophoresis injection. The overlapping loci can be visualized simultaneously by using PCR primers labeled with four different fluorescent tags (see chart on next page).

2.8.11.2 The Applied Biosystems 3130 Genetic Analyzer utilizes electrokinetic injection of DNA molecules into polymer-filled capillaries which separates the DNA fragments by size. The fluorescent tag labeled primers incorporated into the PowerPlex® 16 HS amplification products are responsive to the frequency of the 15 mW argon-ion laser. Upon excitation, the fluorophores are raised to a higher energy level. When the fluorophores return to their normal energy level, a fluorescent signal is emitted. This signal is then detected by a camera within the 3130 capillary electrophoresis instrument which converts the signal to a computer image where it is visualized in an electropherogram as a peak.

2.8.11.3 The data produced by the 3130 Genetic Analyzer is analyzed with GeneMapper® *ID-X* Software which results in peaks labeled with their allele designation. The allele designation for each sample is accomplished through the use of an internal lane standard (ILS). The ILS is injected with each sample and it contains 22 fragments of known length. The ILS determines the base pair size of the fragments in the sample and the software compares the sizes to an allelic ladder to determine the allele designation.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

The PowerPlex ® 16 HS System Locus-Specific and Allelic Ladder Information

Locus	Chromosomal Location	Repeat Sequence[1] 5'-->3'	Allelic Ladder Size Ranges [3,4] (bases)	STR Ladder Alleles [5] (# of repeats)	Fluorophore
Penta E	15q	AAAGA*	379-474	5-24	Fluorescein
D18S51	18q21.3	AGAA*	290-366	8-10, 10.2, 11-13, 13.2, 14-27	Fluorescein
D21S11	21q11-21q21	TCTA*	203-259	24, 24.2, 25, 25.2, 26-28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36-38	Fluorescein
TH01	11p15.5	AATG*	156-195	4-9, 9.3, 10-11, 13.3	Fluorescein
D3S1358	3p	TCTA*	115-147	12-20	Fluorescein
FGA	4q28	TTTC*	322-444	16-18, 18.2, 19, 19.2, 20, 20.2, 21, 21.2, 22, 22.2, 23, 23.2, 24, 24.2, 25, 25.2, 26-30, 31.2, 43.2, 44.2, 45.2, 46.2	TMR
TPOX	2p23-2pter	AATG*	262-290	6-13	TMR
D8S1179	8q	TCTA*	203-247	7-18	TMR
vWA	12p12-pter	TCTA*	123-171	10-22	TMR
Amelogenin[2]	Xp22.1-22.3 and Y	NA	106(X)/112(Y)	X,Y	TMR
Penta D	21q	AAAGA*	376-449	2.2, 3.2, 5, 7-17	JOE
CSF1PO	5q33.3-34	AGAT*	321-357	6-15	JOE
D16S539	16q24-qter	GATA*	264-304	5,8-15	JOE
D7S820	7q11.21-22	GATA*	215-247	6-14	JOE
D13S317	13q22-q31	TATC*	176-208	7-15	JOE
D5S818	5q23.3-32	AGAT*	119-155	7-16	JOE

[1] The August 1997 report (25, 26) of the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) states, "1) for STR loci within coding genes, the coding strand shall be used and the repeat sequence motif defined using the first possible 5´ nucleotide of a repeat motif; and 2) for STR loci not associated with a coding gene, the first database entry or original literature description shall be used".

[2] Amelogenin is not an STR but displays a 106-base, X-specific band and a 112-base, Y-specific band. 9947A DNA (female) displays only the 106-base, X-specific band.

[3] The length of each allele in the allelic ladder has been confirmed by sequence analyses.

[4] When using an internal lane standard, such as the Internal Lane Standard 600, the calculated sizes of allelic ladder components may differ from those listed. This occurs because different sequences in allelic ladder and ILS components may cause differences in migration. The dye label also affects migration of alleles.

[5] For a current list of microvariants, see the Variant Allele Report published at the U.S. National Institute of Standards and Technology (NIST) web site at: **www.cstl.nist.gov/div831/strbase/**

TMR = carboxy-tetramethylrhodamine
FL = fluorescein
JOE = 6-carboxy-4´,5´-dichloro-2´,7´-dimethoxyfluorescein
NA = not applicable

2.8.12 PowerPlex® 16 HS Amplification Set-Up

2.8.12.1 The following steps shall be performed in the PCR amplification set-up area.

2.8.12.2 Thaw the Amplification Grade Water, PowerPlex ® HS 5X Master Mix, and PowerPlex® 16 HS 10X Primer Pair Mix. When thawed, it is important to vortex the PowerPlex® 16 HS 5X Master Mix and PowerPlex® 16 HS 10X Primer Pair Mix tubes for 5 to 10 seconds. (Do not centrifuge the 10X Primer Pair Mix as this may cause the primers to be concentrated at the bottom of the tube.) The Amplification Grade Water may be stored at 4° C for extended periods.

2.8.12.3 Determine the number of samples to be amplified, including controls (reagent blank, positive control and amplification blank). Add 2 to 4 reactions to this number to compensate for the loss that occurs during reagent transfer.

2.8.12.4 Place one 0.2 ml reaction tube for the Model 9700 Thermal Cycler for each sample into a rack and label appropriately. A 96-well plate can also be used if using the robotic workstation.

2.8.12.5 0.5 to 2.0 ng of template DNA is recommended for the amplification. (Amplification of greater than 2.0 ng of template DNA should not be used due to off-scale peak heights and peak height imbalance from locus to locus.) Targeting less than 0.5 ng of template DNA should be used only with samples whose concentration falls between 0.01 ng/µl and 0.028 ng/µl. Such samples shall be interpreted with caution. Samples with concentrations greater than 0.028 ng/µl shall target between 0.5 ng to 2.0 ng for amplification.

2.8.12.6 Using the PP16HS Amplification Worksheet (5 µl template DNA), calculate the required amount of each component of the PCR master mix. Multiply the volume (µl) per sample by the total number of reactions to obtain the final volume (µl).

2.8.12.6.1 Components of Master Mix/sample:

2.8.12.6.2 12.5 µl Nuclease Free or Amplification Grade Water

2.8.12.6.3 5 µl PowerPlex® HS 5X Master Mix

2.8.12.6.4 2.5 µl PowerPlex® 16 HS 10X Primer Pair Mix

2.8.12.6.5 20 µl Total Volume (w/o sample)

2.8.12.7 Add the calculated volume of each component to a 1.5 ml tube. Mix gently.

2.8.12.8 Add 20 µl of PCR master mix to each sample tube or well using a positive displacement pipettor or a repeat pipettor.

2.8.12.9 Pipette 5 µl of each sample into the respective tube or well containing master mix.

2.8.12.10 For samples requiring more than 5 µl of sample volume, use the variable PP16HS Amplification Worksheet to calculate the required amount of each component of the PCR master mix. Sample volumes above 5 µl shall be subtracted from the nuclease free or amplification grade water volume in the master mix.

2.8.12.10.1 Components of the Master Mix/sample:

2.8.12.10.2 Up to 12.5 µl Nuclease Free or Amplification Grade Water

2.8.12.10.3 5 µl PowerPlex® HS 5X Master Mix

2.8.12.10.4 2.5 µl PowerPlex® HS 10X Primer Pair Mix

2.8.12.10.5 Between 7.5 and 20 µl Total Volume (w/o sample)

2.8.12.11 Add the calculated volume of each component to a 1.5 ml tube. Mix gently.

2.8.12.12 Add up to 20 µl of PCR master mix to each sample tube or well using a positive displacement pipettor or a repeat pipettor.

2.8.12.13 Pipette the appropriate amount of each sample (up to 17.5 µl) into the respective tube or well containing master mix. For organic extractions if the template DNA is stored in TE buffer, the volume of the DNA sample added should not exceed 20% of the final reaction volume. PCR amplification efficiency and quality can be greatly altered by changes in pH (due to added Tris-HCl) or available magnesium concentration (due to chelation by EDTA). DNA samples stored (or diluted) in NFH_2O are not subject to this caution, but may contain other PCR inhibitors at low concentrations depending on the source of the template DNA and the extraction procedure employed.

2.8.12.14 For the positive control, dilute the 9947A, the 2800M or other approved positive DNA standard supplied with the PowerPlex® 16 HS kit to 0.1 - 0.3 ng/µl. Pipette 5.0 µl (0.5 to 2.0 ng) of diluted DNA into a microcentrifuge reaction tube or well containing 20 µl of PCR master mix. A positive control shall be included in each thermal cycler.

2.8.12.15 For the negative amplification control, pipette 5µl of nuclease free or amplification grade water into a microcentrifuge reaction tube or well containing 20µl of the PCR master mix. A negative amplification control shall be included in each thermal cycler.

2.8.13 PowerPlex® 16 HS Amplification

2.8.13.1 Assemble the tubes or place the 96-well plate in a thermal cycler.

2.8.13.2 Select and run the appropriate thermal cycling protocol.

2.8.13.3 Applied Biosystems GeneAmp® 9700 Thermal Cycler

2.8.13.3.1 96°C for 2 minutes, then:

2.8.13.3.2 Ramp 100% to 94°C for 30 seconds

2.8.13.3.3 Ramp 29% to 60°C for 30 seconds

2.8.13.3.4 Ramp 23% to 70°C for 45 seconds

2.8.13.3.5 for **10 cycles**, then:

2.8.13.3.6 Ramp 100% to 90°C for 30 seconds

2.8.13.3.7 Ramp 29% to 60°C for 30 seconds

2.8.13.3.8 Ramp 23% to 70°C for 45 seconds

2.8.13.3.9 for **20 cycles**, then:

2.8.13.3.10 60°C for 30 minutes, then:

2.8.13.3.11 Soak at 4°C until the tubes are removed

2.8.13.4 When programming the GeneAmp® PCR System 9700 thermal cycler, use the ramping mode for the GeneAmp® PCR System 9600 thermal cycler.

2.8.13.5 Select 25 µl for the volume in the amp tubes or wells.

2.8.13.6 Remove samples after the amplification process is completed.

2.8.13.7 Store the amplified samples in the freezer or refrigerator (if they are to be used within 2 days).

2.8.14 Applied Biosystems 3130 Genetic Analyzer - Data Collection Software version 3.0 - Instrument Set-up

2.8.14.1 Creating a Run Module, Protocol, and Results Group (**only necessary the first time PowerPlex® 16 HS samples are run or if the parameters change**.)

2.8.14.2 In the "Module Manager," select "New." Select "Regular" in the "Type" drop-down list, and select "HIDFragmentAnalysis36_POP4" in the "Template" drop-down list. Confirm that the injection time is 5 seconds and the injection voltage is 3 kV. Lengthen the run time to 2,000 seconds. Name the run module with the kit name, injection voltage and injection time and select "OK". Additional validated injection protocols may be utilized. The acceptable injection protocols shall be listed within the Indiana State Police Laboratory Forensic Biology Section Casework Test Method under *General Rules for PowerPlex® 16 HS Analysis on the Applied Biosystems 3130 Genetic Analyzer*. (Injection protocols Hot Start) A run module and instrument protocol shall be created for each injection time protocol needed.

2.8.14.3 In the "Protocol Manager," select "New." Name the protocol with the kit name, injection voltage and injection time. Select "Regular" in the "Type"

drop-down list, and select the appropriate run module from the "Run Module" drop-down list. Select "F" in the "Dye-Set" drop-down list. Select "OK."

2.8.14.4 In the "Results Group Manager", select "New." Select the "General" tab and enter "PowerPlex® 16" for the name. Select the "Analysis" tab, and select "GeneMapper—Generic" in the "Analysis Type" drop-down list. Leave the destination as "E:\Applied Biosystems\udc\datacollection\data." Select the "Naming" tab and change the Prefix to "PP16". Under Format, select "Well Position" and "Sample Name" in the drop-down lists. Under the options for the Run Folder Name, select "Plate Name" from the drop-down lists. Under the Automated Processing tab select that Autoanalysis is performed "Only when the results group is complete."

2.8.15 Applied Biosystems 3130 Genetic Analyzer - Data Collection Software version 3.0 - PowerPlex® 16 HS Electrophoresis

2.8.15.1 Sample Preparation

2.8.15.1.1 **Note:** The quality of formamide is critical for the successful detection of a DNA profile. Deionized formamide shall be used that has a conductivity of less than 100µS/cm, such as Hi-Di™ Formamide. The formamide shall be frozen in aliquots at -20^{o}C and the remainder of each aliquot shall be discarded after it is thawed. Multiple freeze-thaw cycles or long-term storage at 4^{o}C may cause breakdown of the formamide which can create ions that compete with DNA during injection. This will cause lower peak heights and decreased sensitivity.

2.8.15.1.2 **Caution:** Formamide is an irritant and teratogen; therefore universal precautions and a fume hood shall be utilized when manually working with formamide to avoid inhalation and contact with the skin.

2.8.15.1.3 Thaw the ILS 600, the allelic ladder, and an aliquot of Hi-Di™ Formamide. When thawed, vortex to mix.

2.8.15.1.4 Determine the number of samples to be injected, including controls (reagent blanks, positive control and amplification blank) and allelic ladders. Add 2 to 4 reactions to this number to compensate for the loss that occurs during reagent transfers.

2.8.15.1.5 Prepare a loading cocktail by combining the internal lane standard (ILS 600) with the Hi-Di™ Formamide as follows:

2.8.15.1.6 [(1 µl ILS 600) x (# samples)] + [(9 µl Hi-Di™ Formamide) x (# samples)]

2.8.15.1.7 The volume of ILS 600 used in the loading cocktail may be decreased to optimize size standard peaks. The optimal range of peak heights for the size standard should be ~ 400 to 1000 RFU. If the peak heights of the size standard are too high, pull-up of the size standard peaks into the other dyes can occur. Optimization of size standard peaks to limit the observation of pull-up while maintaining sufficient peak heights can be obtained by using 0.5 to 1.0 µl of ILS

600 per well. The amount of Hi-Di™ Formamide shall be adjusted so that the total amount of loading cocktail for each well is 10 µl.

2.8.15.1.8 Vortex to mix.

2.8.15.1.9 Pipette 10 µl of the formamide/ILS 600 mixture into each well. (Add formamide or formamide/ILS mixture into empty wells to complete an injection set of four or sixteen. Every well in which an injection is occurring must contain liquid.) Alternatively, the robot workstation may be utilized.

2.8.15.1.10 Add 1 µl of amplified sample or 1 µl of the allelic ladder mix to each well. It is recommended that one allelic ladder is injected within each set of 16 samples on a four capillary instrument and within every 32 samples on a sixteen capillary instrument to ensure that a usable ladder injection occurs. At least one allelic ladder is required within each run folder.

2.8.15.1.11 Cover the wells with the plate septa and briefly spin down to remove air bubbles from the wells.

2.8.15.1.12 Denature the samples at 95^{o}C for ~3 minutes, then immediately chill on crushed ice or a cold pack for ~3 minutes. Denature the samples just prior to loading the instrument. Avoid denaturing the samples for longer than 3 minutes as extended heat denaturing can lead to the appearance of artifacts.

2.8.15.2 Creating a Plate Record

2.8.15.2.1 In the “Plate Manager,” select “New.” Name the plate record “Laboratory case number_date_ the injection number of the plate” (ex. 00A1234_01Jan11_01). If more than one laboratory case number is loaded onto a plate, use the first file number injected in the plate name. The first injection of a prepared plate shall be 01, if the plate is re-injected the number shall increase sequentially. If the same plate preparation is run on a different date, the original date should still be used from when the plate was prepared. If a second preparation of a plate is made on the same date as the first, it shall be designated with a “-2” after the date. (ex. 00A1234_01Jan11-2_01) In the “Application” drop-down list select “GeneMapper—Generic”, and select 96-well in the plate type drop-down list. Add your name or initials in the owner and operator windows, and select “OK”.

2.8.15.2.2 In the plate record, enter sample names in the appropriate cells and scroll to the right. The sample name shall include the sample sub-item as well as the laboratory case number (ex. 1A1_00A1234) if more than one case is included on the plate. If only one case is present on the plate then only the subitem number is required. In order to aid in GeneMapper® *ID-X* sample analysis, it is recommended to place a “z” in front of the sample name for known standards (ex. z1A1_00A1234”) here or in Genemapper *ID-X* under sample name. In the “Results Group 1” column, select the “PowerPlex16” results group from the drop-down list. In the

"Instrument Protocol 1" column select the appropriate protocol from the drop-down list. Fill in these selections for each row that contains a sample name. The instrument will inject in groups of 4 or 16 (depending on the number of capillaries) regardless of whether there is a sample name. Be sure all wells in a group have Hi-Di™ Formamide present.

2.8.15.2.3 If more than one run is needed for any given sample, select "Edit", then "Add Sample Run" and fill in the appropriate Results Group and Instrument Protocol entries. If only specific samples are to be run with a second protocol, only those samples to be analyzed need to contain a Results Group and an Instrument Protocol.

2.8.15.2.4 Fill out a Plate Record Worksheet for each plate. Copies of the plate record worksheet shall be maintained in the case record.

2.8.15.3 Starting the Plate Run

2.8.15.3.1 Check that daily maintenance activities have been performed (i.e. buffer and dH_2O reservoirs have been rinsed and re-filled) and that no bubbles are present in the polymer delivery area. If bubbles are present, run the "Bubble Remove Wizard."

2.8.15.3.2 Place the prepared and assembled plate on the autosampler.

2.8.15.3.3 In the "Run Scheduler", click "Find All" to bring up the list of plate records. To link the plate to the plate record, select the plate record from the list, then click the plate position indicator. The plate position indicator will change from yellow to green. Click the green "Run Instrument" arrow, and then click "OK" in the start processing dialog box.

2.8.15.3.4 Record the run information in the 3130 Run Log.

2.8.15.3.5 When the run is complete, remove the plate from the instrument and store in the freezer until you have checked all of the sample results to ensure that no samples need to be re-injected.

2.8.16 Setting Up GeneMapper® *ID-X* Version 1.2 Software User Accounts and the Security System

2.8.16.1 Setting Up Password Policies

2.8.16.1.1 Open the GeneMapper® ID-X version 1.2 software.

2.8.16.1.2 Login using an administrator user account.

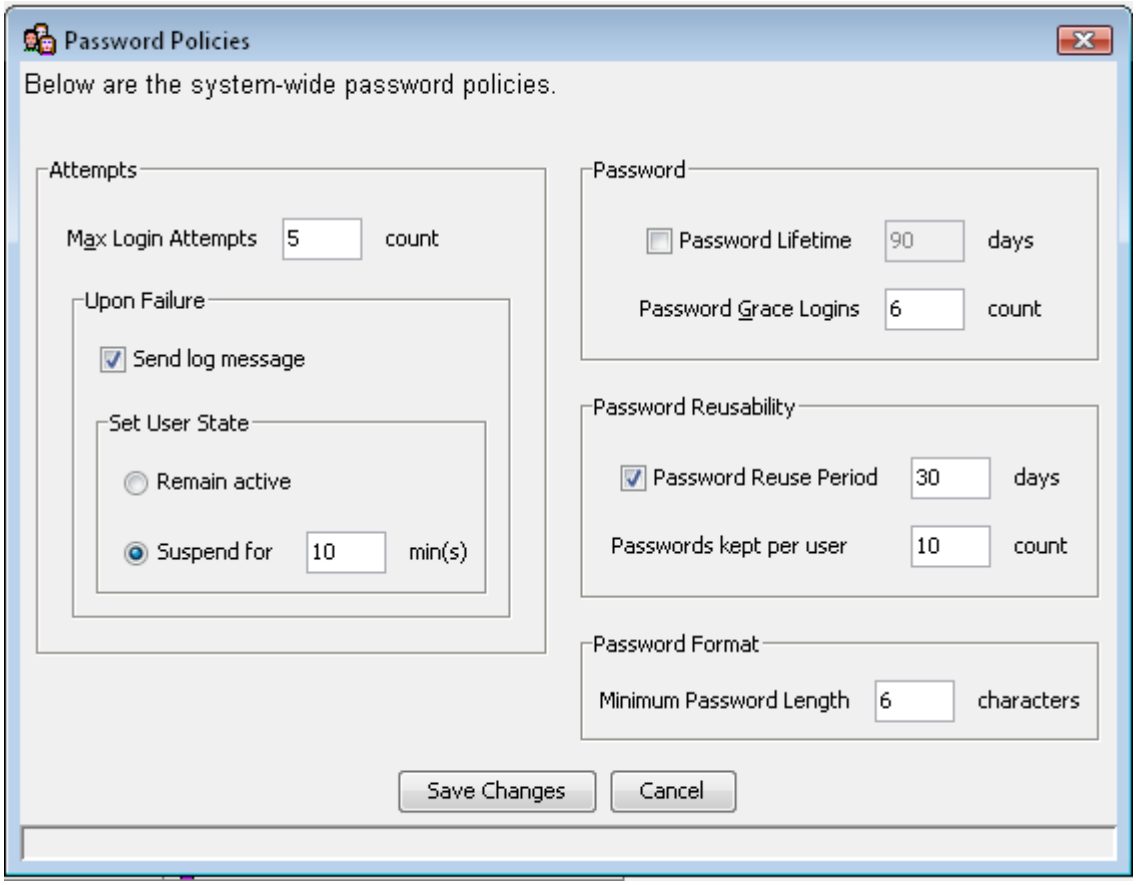
2.8.16.1.3 Select "Admin" then "Security Manager."

2.8.16.1.4 Login with the same administrator user account.

2.8.16.1.5 Select "Settings" then "Password Policies."

2.8.16.1.6 Change the settings to match Figure 1.

Figure 1: Password Policies



2.8.16.1.7 Save changes.

2.8.16.2 Creating a User Group

2.8.16.2.1 Open the Security Manager.

2.8.16.2.2 Select the appropriate default user group in the left navigation pane (e.g. “Casework User Group”).

2.8.16.2.3 Select “Edit” then “Duplicate.”

2.8.16.2.4 In the “General” section, enter a name for the user group (e.g. “ISP Casework User Group”).

2.8.16.2.5 In the “Default Rights” section, make sure the “Read” and “Update” options are checked.

2.8.16.2.6 Select the “Security Groups” tab. Ensure all associated security groups are checked, including the “GeneMapper ID-X” security group.

2.8.16.3 Creating a Security Group

2.8.16.3.1 Open the Security Manager.

2.8.16.3.2 Select the appropriate default security group in the left navigation pane (e.g. “Casework Security Group”).

2.8.16.3.3 Select “Edit” then “Duplicate.”

2.8.16.3.4 In the “General” section, enter a name for the security group (e.g. “ISP Casework Security Group”).

2.8.16.3.5 In the “Associate” column, select the appropriate user groups to associate with that security group (e.g. “ISP Casework User Group”). Make sure the Read and Update options are checked for the selected user groups.

2.8.16.4 Creating a Profile

2.8.16.4.1 Open the Security Manager.

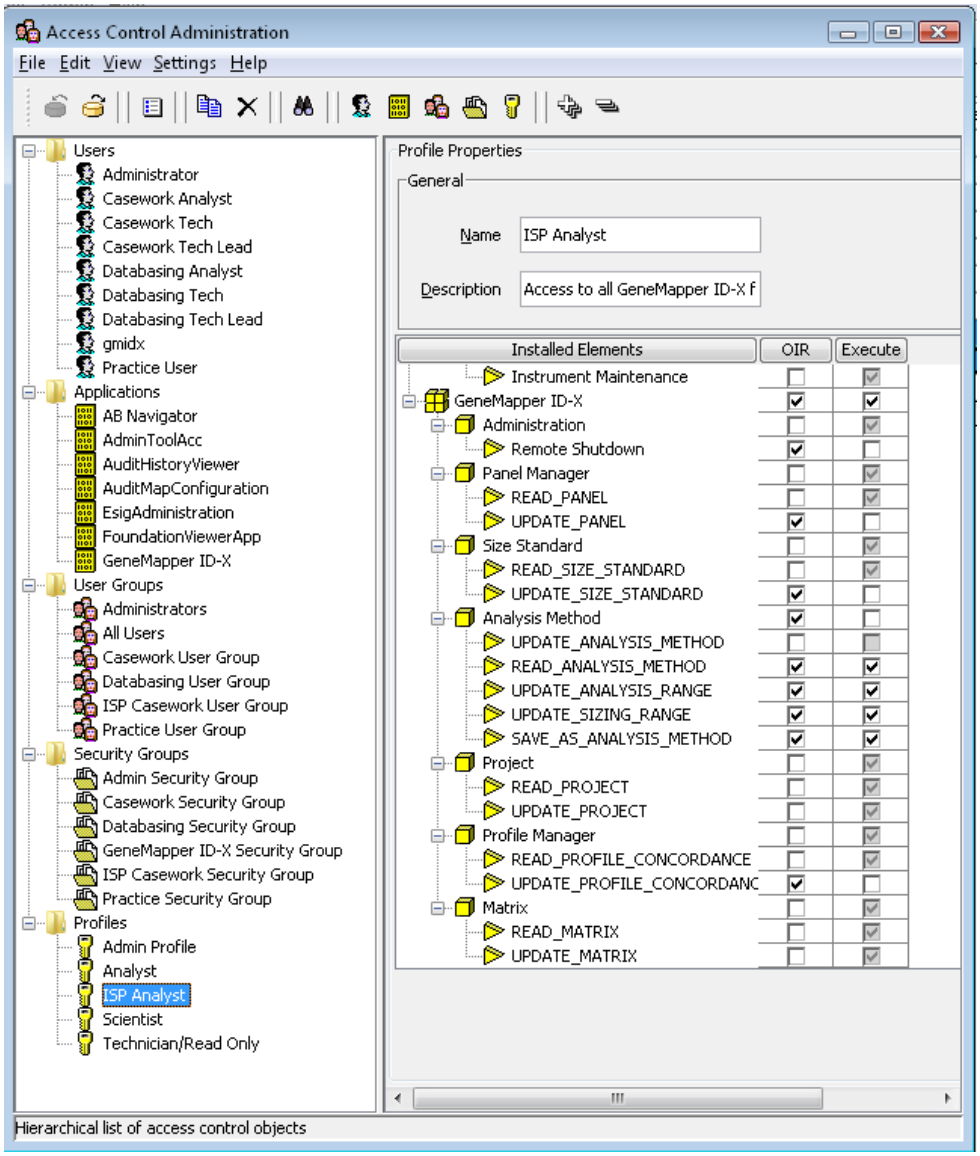
2.8.16.4.2 Select the appropriate default profile in the left navigation pane (e.g. “Analyst”).

2.8.16.4.3 Select “Edit” then “Duplicate.”

2.8.16.4.4 In the “General” section, enter a name for the profile (e.g. “ISP Analyst”).

2.8.16.4.5 In the “Installed Elements” table, select allowed actions for that particular profile. Allowed actions are not automatically inherited from the default profile and have to be manually entered. For the “ISP Analyst” profile, check the same allowed actions as those for the default GeneMapper® *ID-X* “Analyst” profile, with the exception of the “Update Panel” and “Update Size Standard” actions. Change those actions to match the selections in Figure 2.

Figure 2: Allowed Actions of the "ISP Analyst" Profile



2.8.16.5 Setting Up A User Account

2.8.16.5.1 Open the "Security Manager."

2.8.16.5.2 Select the appropriate default user type in the left navigation pane for that particular user (e.g. "Casework Analyst").

2.8.16.5.3 Select "Edit" then "Duplicate."

2.8.16.5.4 In the "General" section, enter a name. This will be the login name.

2.8.16.5.5 In the "User Details" section, enter a full name. Leave the status set to "Active." Uncheck "Show EULA."

2.8.16.5.6 Select a profile (e.g. "ISP Analyst").

2.8.16.5.7 Select one or more user groups (e.g. “ISP Casework User Group”).

2.8.16.5.8 In the “Password” section, select “Set Password.” Enter the same password twice and click “OK.” Check “Pre-Expire.”

2.8.17 GeneMapper® *ID-X* Version 1.2 Software - PowerPlex® 16 HS Software Settings

2.8.17.1 Importing Panel and Bin Files

2.8.17.1.1 Open the GeneMapper® *ID-X* version 1.2 software.

2.8.17.1.2 Select “Tools” then “Panel Manager”.

2.8.17.1.3 Highlight the “Panel Manager” icon in the navigation pane.

2.8.17.1.4 Select “File” then “Import Panels”.

2.8.17.1.5 Navigate to the saved panel, bin, and stutter files. Select “PowerPlex16HS_ISPv2_IDX1.2.0_Panels.txt” then click “Import”.

2.8.17.1.6 Select the “ISP Casework Security Group.” Click “OK.”

2.8.17.1.7 In the navigation pane, highlight the “PowerPlex16HS_ISPv2_IDX1.2.0_Panels” folder.

2.8.17.1.8 Select “File” then “Import Bin Set”.

2.8.17.1.9 Select “PowerPlex16HS_ISPv2_IDX1.2.0_Bins.txt” then click “Import”.

2.8.17.1.10 In the navigation pane, highlight the “PowerPlex16HS_ISPv2_IDX1.2.0_Panels” folder.

2.8.17.1.11 Select “File” then “Import Marker Stutter.” A warning box will appear asking to overwrite the current values. Select “Yes”.

2.8.17.1.12 Select “PowerPlex16HS_ISPv2_IDX1.2.0_Stutter.txt” then click “Import.” This will import the Promega Marker Stutter file that has been modified to include the Indiana State Police PowerPlex® 16 HS stutter percentages for filtering out stutter as determined by internal validation studies.

2.8.17.1.13 In the Panel Manager window, select “Apply”, then “OK”.

2.8.17.2 Creating a Casework Analysis Method

2.8.17.2.1 Select “Tools”, then “GeneMapper® *ID-X* Manager”.

2.8.17.2.2 Select the Analysis Methods tab.

2.8.17.2.3 Select “New” and a new analysis method dialog box will open.

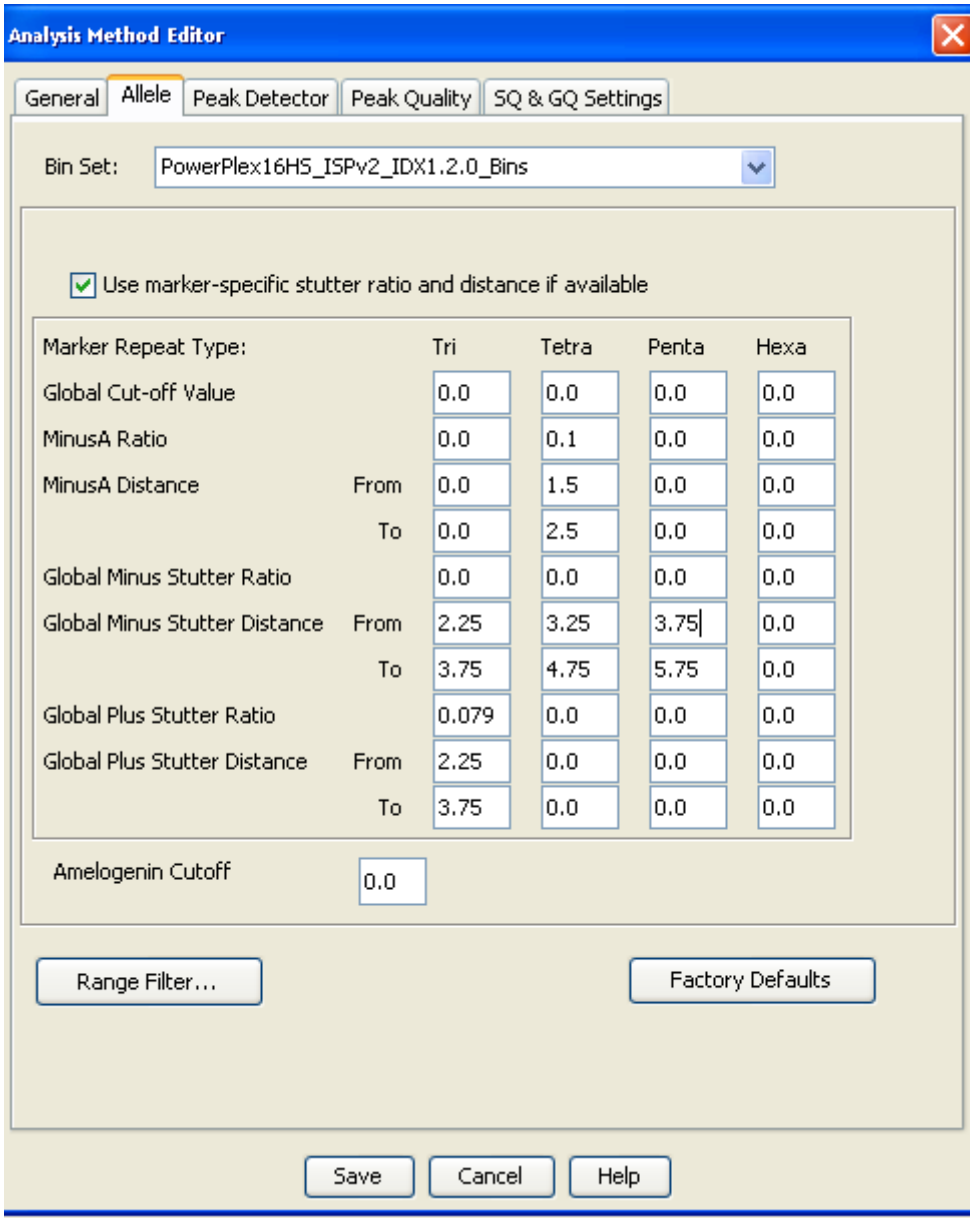
2.8.17.2.4 Enter the name “PP16 HS”.

2.8.17.2.5 Select the “ISP Casework Security Group”.

2.8.17.2.6 Enter 3130 as the instrument.

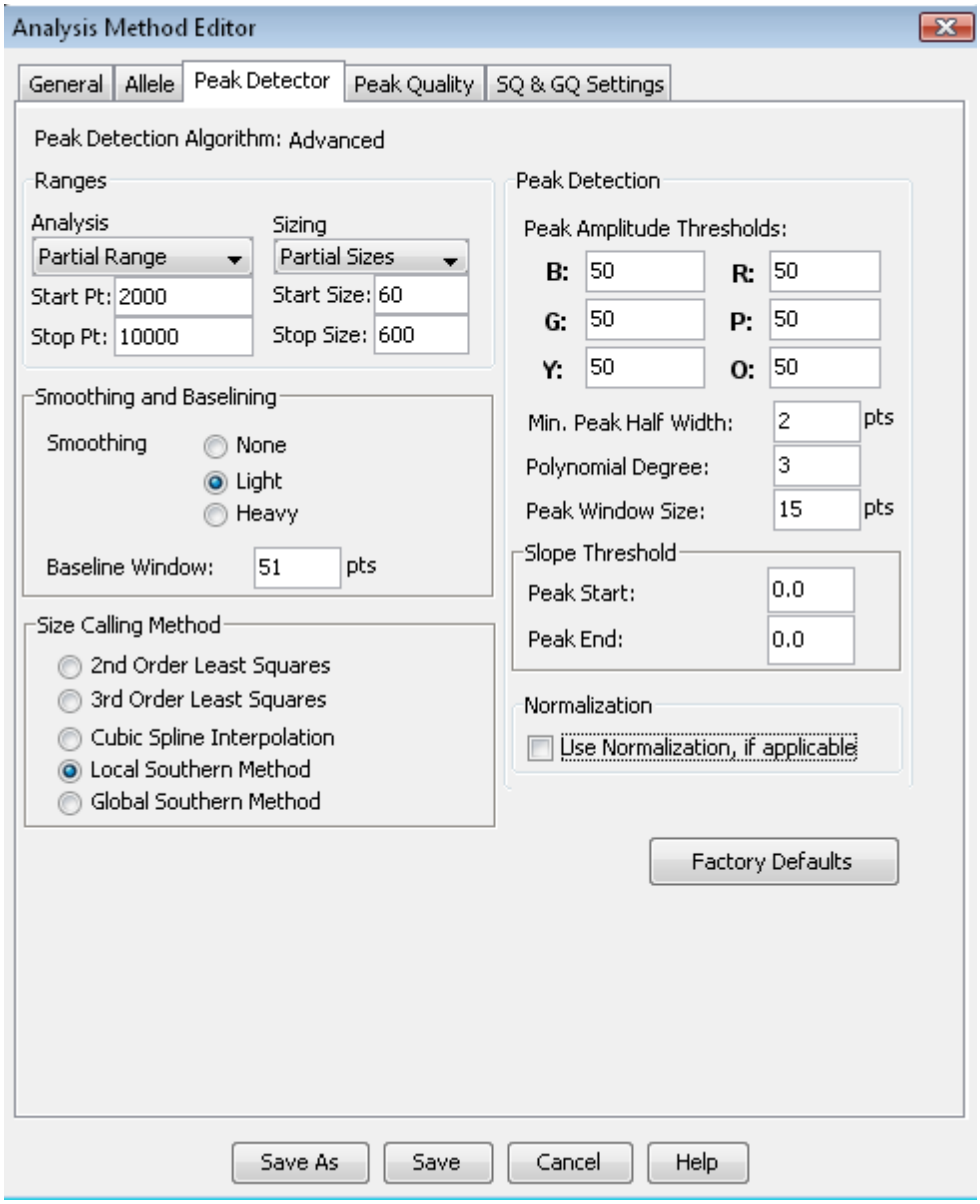
2.8.17.2.7 Select the “Allele” tab. In the “Bin Set” drop-down menu select “PowerPlex16_ISPv2_IDX1.2.0_Bins”. Ensure that the “Use marker-specific stutter ratio if available” box is checked. Enter the values shown in Figure 1 for proper filtering of stutter peaks.

Figure 1: The Allele Tab.



2.8.17.2.8 Select the “Peak Detector” tab. Change the settings to match Figure 2. Alternatively, the Analysis Range may be set to “Full Range” if the Instrument Migration warrants it (data coming off late).

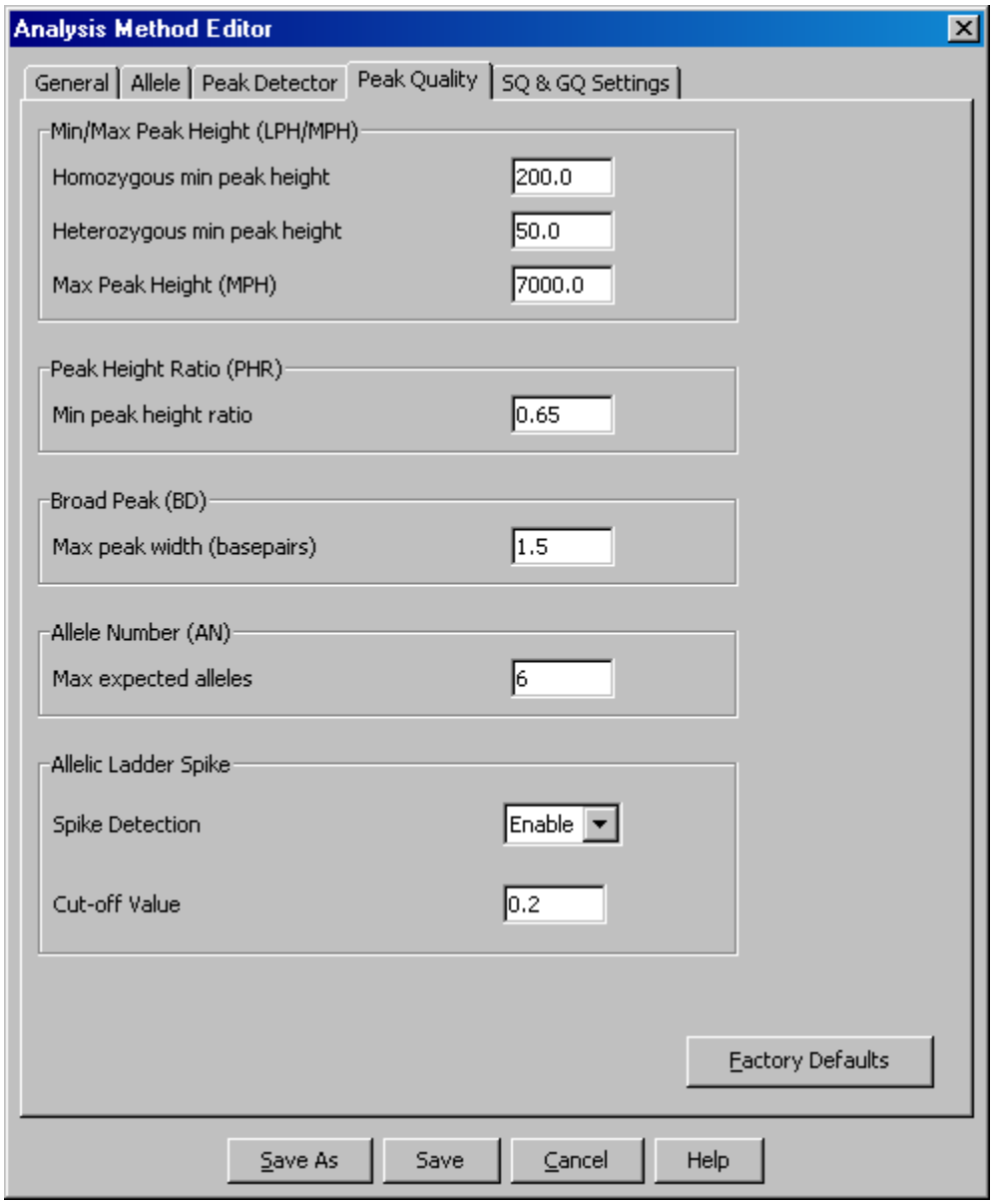
Figure 2: The Peak Detector Tab.



2.8.17.2.9 The Peak Amplitude Threshold (analytical threshold) values were determined to be 50 RFU for PowerPlex® 16 HS 30 cycle amplification. The analytical threshold value should be calculated during validation for each 3130 instrument in the Indiana State Police laboratory system and may vary. If different threshold values are used, they must be posted on the instrument with the documented approval of the Technical Leader entered into the maintenance log.

2.8.17.2.10 Select the "Peak Quality" tab. Change the settings to match Figure 3.

Figure 3: The Peak Quality Tab.



2.8.17.2.11 Leave the “SQ and GQ Settings” tab set to the factory defaults.

2.8.17.2.12 Select “Save.”

2.8.17.3 Creating a Size Standard

2.8.17.3.1 Select “Tools”, then “GeneMapper Manager”.

2.8.17.3.2 Select the “Size Standard” tab and click “New”.

2.8.17.3.3 Name the size standard “ILS HS” in the “Size Standard Editor” screen, choose the “ISP Casework Security Group”, and choose red as the color for the size standard dye.

2.8.17.3.4 Enter the sizes of the 21 allelic ladder fragments: 60, 80, 100, 120, 140, 160, 180, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, and 550bp.

2.8.17.3.5 Select "OK".

2.8.17.4 Creating a Table Setting

2.8.17.4.1 Select "Tools", then "GeneMapper Manager".

2.8.17.4.2 Select the "Table Setting" tab and click "New".

2.8.17.4.3 Under the "General" tab name the Table Setting "PP16 HS" and select the "ISP Casework Security Group".

2.8.17.4.4 Under the "Samples" tab ensure that check marks are located next to only the following: Status, Sample File, Sample Name, Sample Type, Analysis Method, Panel, Size Standard, Sizing Quality Overridden, Sample File Not Found, Sample Off-scale, and Sizing Quality. Leave the font "Arial" and the size 11. Sort by "Sample Type" and then by "Sample Name." Select the "Ascending" option for both, and leave the final sort option to "None."

2.8.17.4.5 Under the "Genotypes" tab ensure that check marks are located next to only the following: Sample File, Sample Name, Panel, Marker, Dye, Allele, Size, Height, Off-scale, Out of Bin Allele, Peak Height Ratio, Control Concordance, and Genotype Quality. Sort by "Sample Name," then by "Marker," and then by "Sample File." Select the "Ascending" option for all three. Also, change the "Show number of alleles" to 8 and check "Keep Allele, Size, Height, Area, Data Point, Mutation and Comment together". (If the option to "Keep Allele, Size, Height…" is not visible in the window, expand the window size until the option appears.) Leave the font "Arial" and the size 11.

2.8.17.4.6 Select "OK."

2.8.17.5 Creating a Plot Setting – Samples, Controls, and Ladders

2.8.17.5.1 Select "Tools", then "GeneMapper Manager".

2.8.17.5.2 Select the "Plot Settings" tab and click "New".

2.8.17.5.3 Under the "General" tab name the Plot Setting "PP16 HS" and select the "ISP Casework Security Group".

2.8.17.5.4 Under the "Sample Header" tab ensure that check marks are located next to the following: Sample File, Sample Name, Panel, Sizing Quality Overridden, Sample Off-Scale, and Sizing Quality.

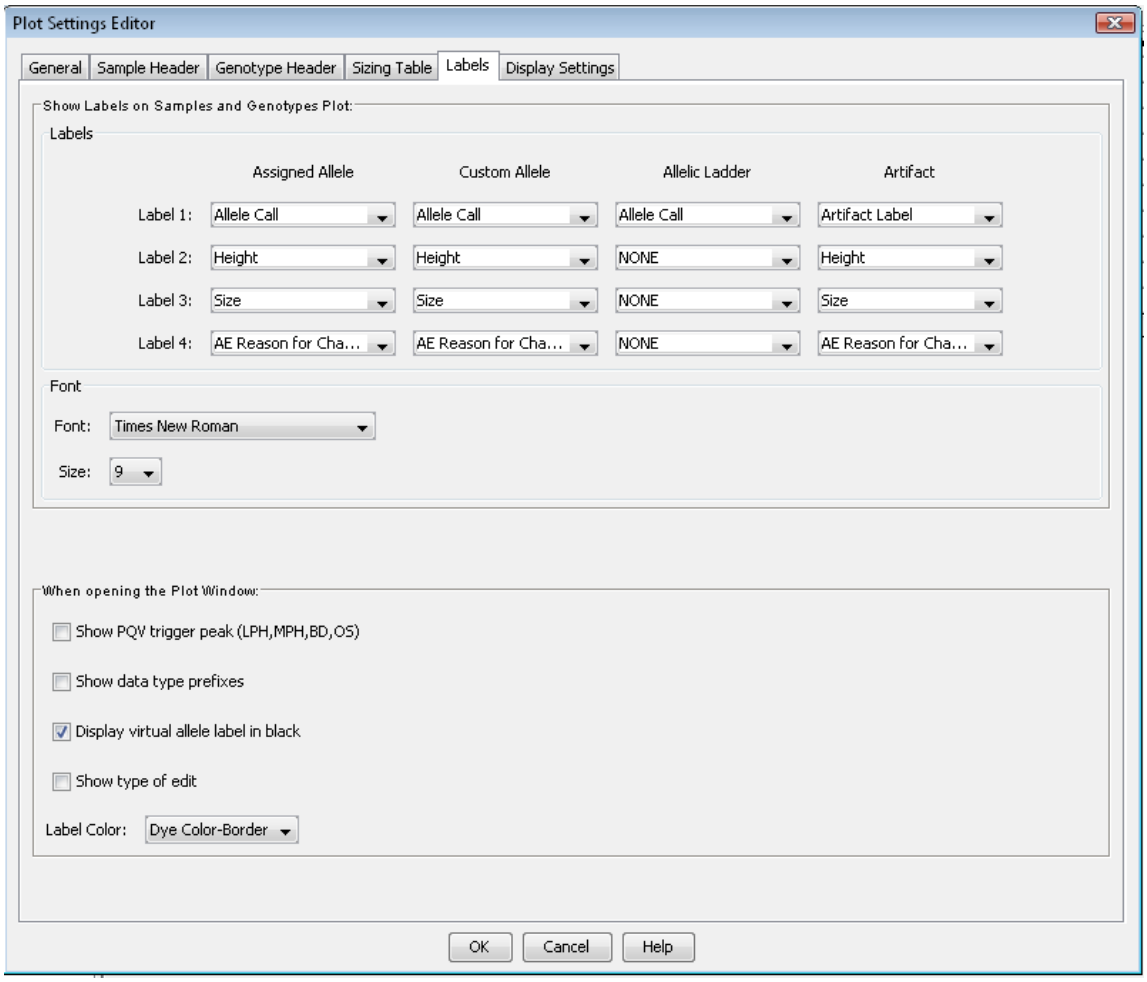
2.8.17.5.5 Under the "Genotype Header" tab ensure that check marks are located next to the following: Sample File, Sample Name, Panel, Marker, Off-scale, Out of Bin Allele, Peak Height Ratio, Control Concordance, and Genotype Quality.

2.8.17.5.6 Under the "Sizing Table" tab ensure that check marks are located next to the following: Dye/Sample Peak, Sample File Name, Marker,

Allele, Size, Height, Area, and Data Point. Leave the font "Arial" and the size 11.

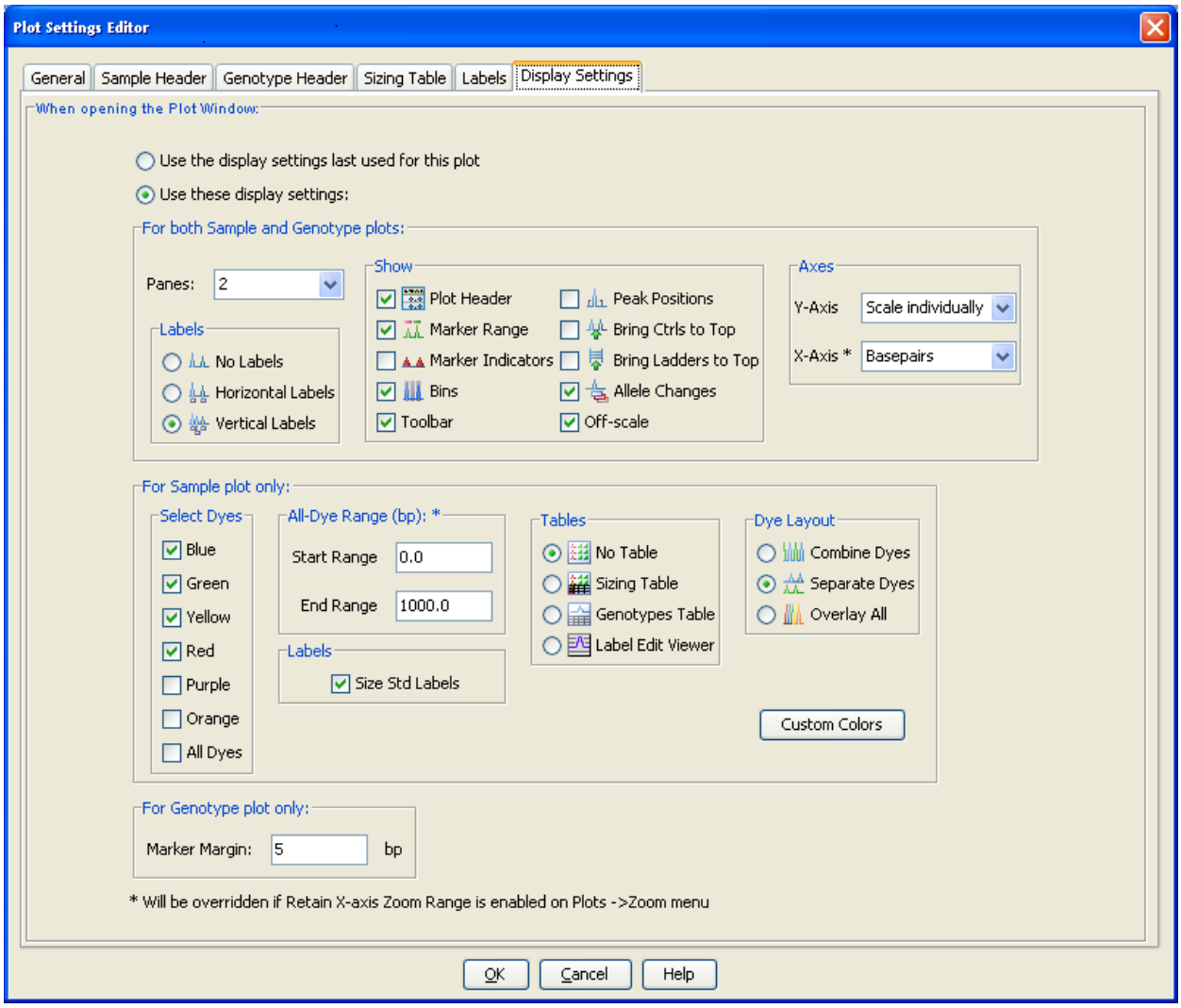
2.8.17.5.7 Select the "Labels" tab. Change the settings to match Figure 4. Alternatively, if the "allele edit comment" function is not being used, "Label 4" for "Assigned Allele", "Custom Allele" and "Artifact" can be changed to "NONE".

Figure 4: The Labels Tab.



2.8.17.5.8 Under the "Display Settings" tab, change the display settings so that they match Figure 5.

Figure 5: The Display Settings Tab.



2.8.17.5.9 Select "OK."

2.8.18 GeneMapper® *ID-X* Version 1.2 Software - PowerPlex® 16 HS Data Analysis

2.8.18.1 Processing Sample Data

2.8.18.1.1 Import the sample files from a single run folder by "Edit", then selecting "Add Samples to Project".

2.8.18.1.2 In the "Add Samples to Project" screen, navigate to the run folder that contains the sample files. If the entire run folder is to be imported, click on the folder to highlight it; then click the "Add to List" button at the bottom of the window. If only a portion of samples need to be selected, expand the folder to view the samples. Highlight the appropriate samples, ensuring that the allelic ladder and all the desired samples are selected. Once all the samples are selected click the "Add to List" button at the bottom of the window.

2.8.18.1.3 Only one injection parameter per project. A run folder shall not be created manually by manipulating sample files.

2.8.18.1.4 Ensure that the necessary files are now located in the "Samples to Add" window by double-clicking on the folder in the right pane, then click "Add".

2.8.18.1.5 After the samples have been added to the project, first briefly scan the raw data to ensure that a bad injection did not occur. To check the raw data, first expand the project folder in the left navigation pane, then click on a sample file, then click on the "Raw Data" tab in the right GeneMapper® window. To return to the "Samples" window, click on the project folder at the top of the left navigation pane.

2.8.18.1.6 The GeneMapper® *ID-X* project shall contain at least one allelic ladder from each run folder included in the project for proper genotyping. Multiple allelic ladders within a run folder will be averaged by the software to calculate the allelic bins. If a ladder injection is of low quality, delete the ladder or change the sample type from "Allelic Ladder" to "Sample" to remove it from consideration in calculating the bins.

2.8.18.1.7 Ensure that the table setting at the top of the screen is set to "PP16 HS".

2.8.18.1.8 In the "Sample Type" column, use the drop-down menu to select "Allelic Ladder", "Sample", "Positive Control" or "Negative Control" for each sample.

2.8.18.1.9 In the "Analysis Method" column, for each sample select "PP16 HS" from the drop-down menu. Click the column header cell to highlight the entire column, then select "Edit", then "Fill Down" (or the shortcut Ctrl + D).

2.8.18.1.10 In the "Panel" column, for each sample select "Promega_ISPHSv2_IDX1.2.0_Panels" from the drop-down menu. Click the column header cell to highlight the entire column, then select "Edit", then "Fill Down" (or the shortcut Ctrl + D).

2.8.18.1.11 In the "Size Standard" column, select "ILS HS" from the drop-down menu. Click the column header cell to highlight the entire column, then select "Edit", then "Fill Down" (or the shortcut Ctrl + D).

2.8.18.1.12 The Analysis Method, Size Standard, and Panel can be set as defaults when a GeneMapper® *ID-X* project is opened. Under the "File" menu, select "Project Options". Under the "Add Samples" tab select the above settings as the default in the drop-down menus for Analysis Method, Size Standard, and Panel. Click "OK."

2.8.18.1.13 Select the green "Analyze" arrow button to start the data analysis. At the Project name prompt, save the project. At a minimum the project name shall contain the injection parameters for the project and date the sample run was started on the instrument. The case number

is recommended but not required. Select the "ISP Casework Security Group."

2.8.18.2 Evaluating Sample Data

2.8.18.2.1 The Sizing Quality shall be at least 0.75 for it to pass and should be close to 1.0.

2.8.18.2.2 Highlight all sample rows containing Allelic Ladders. Then click "View", then "Display Plots". In the Samples Plot screen, change the "Plot Setting" drop-down box to "PP16 HS". Magnify the area from about 100 bp to 500 bp. Then click "File">"Print">"Print" to print off the allelic ladder electropherograms. Check the allelic ladders to ensure that the correct allele calls are made for each peak. (Refer to the PowerPlex®16 HS System Technical Manual for current Allelic Ladder allele calls.) Close out of the Samples Plot window.

2.8.18.2.3 Highlight all sample rows containing negative controls (ex. amplification blanks and reagent blanks). Then click "View", then "Display Plots". In the Samples Plot screen, change the "Plot Setting" drop-down box to "PP16 HS". Print off the entire electropherogram ensuring that the primer peak is visible by clicking "File">"Print">"Print" to print off the negative control electropherograms. Check the negative controls to ensure that no peaks above threshold are present. Close out of the Samples Plot window.

2.8.18.2.4 Highlight all remaining sample rows. Then click "View", then "Display Plots". In the Samples Plot screen, change the "Plot Setting" drop-down box to "PP16 HS". Magnify the area from approximately 100 bp to 500 bp. After evaluating all allele calls, click "File">"Print">"Print" to print off all sample electropherograms. Optionally, the remaining sample rows may be viewed, evaluated, and printed with the Allelic Ladders.

2.8.18.2.5 After all analysis is complete, save the 3130 Data Collection Run Folder containing the sample files and associated GeneMapper® *ID-X* projects under each associated laboratory case number and request folder located in the analysts' folders on the server. Projects should be deleted monthly from the "GeneMapper® *ID-X* Manager" to maintain database space.

2.8.18.2.5.1 When exporting the GeneMapper® *ID-X* project, ensure that the "Export with analysis settings" box is checked.

2.8.18.2.6 The number of audit records on the GeneMapper® *ID-X* database should be routinely checked. Audit records should be backed-up, saved to the DNA server, and then deleted from the GeneMapper® *ID-X* database monthly or if the number of records exceeds 40,000. If the number of audit records exceeds 60,000, the performance of the software may be affected.

2.8.18.2.7 The amount of database space in the GeneMapper® *ID-X* software should be routinely checked. If the occupied space exceeds 80%, additional disk space should be allocated.

2.8.19 General Rules For Powerplex® 16 HS Analysis On The Applied Biosystems 3130 Genetic Analyzer

2.8.19.1 At least one allelic ladder shall be present within a run folder. It is recommended that an allelic ladder be run within each set of 16 wells on a four capillary instrument and within every 32 samples on a sixteen capillary instrument. This will help account for possible migration shifts due to external environmental factors.

2.8.19.2 The placement of unknown samples in the 96-well plate should be done so that the orientation allows for the injection of unknown samples prior to the injection of any standards for that case.

2.8.19.3 An extended pre-run time injection protocol may be used. The pre-run time may be increased from 180 to 1000 seconds. An extended pre-run time may be beneficial if split peaks or excessive shouldering of peaks is observed.

2.8.19.4 A signal range of 1,000 to 2,000 RFU will produce optimal results. Optimization of signal can be obtained by changing the injection parameters. The allowable injection parameters are 3kV 3 second, 3kV 5 second, or 3kV 8 second injection.

2.8.19.4.1 An additional means of decreasing the peak height would be to decrease the sample amount loaded to 0.5 µl. This shall require approval of the Technical Leader documented in the case record, with notification of results forwarded to Technical Leader. Decreasing the sample amount loaded can result in uneven peak heights across loci, therefore these sample results shall be interpreted with caution.

2.8.19.4.2 An additional means of increasing the peak height would be to increase the amount of sample loaded up to 2 µl. This shall require approval of the Technical Leader documented in the case record, with notification of all results given to Technical Leader. Other methods of sample optimization should be attempted (i.e. sample clean-up or concentration) before these measures are taken. The results of these measures shall be interpreted with caution due to possible allelic drop-out, stochastic effects, or other possible factors associated with amplifying low amounts of template DNA.

2.8.19.4.2.1 **NOTE:** Loading more than 1 µl will make the sample ineligible for entry into CODIS.

2.8.19.5 If a sample is to be re-injected at higher injection parameters, the reagent blank and the amplification blank associated with that sample shall also be re-injected at the higher parameters. The positive control need not be injected at the same parameters as the samples associated with it.

2.8.19.6 If a selection of samples from an amplification requires re-injection at higher injection parameters with their associated blanks and the blanks at the higher parameters demonstrate some indication of contamination, the Technical Leader shall be informed. The decision to declare inconclusive all sample data or just the sample data from the higher injection parameter shall be at the discretion of the analyst with the approval of the Technical Leader documented in the case record.

2.8.19.7 If multiple amplifications or injection protocols are analyzed for a sample, the analyst shall use the amplification and/or injection which they determine sufficiently represents the sample based on peak heights, artifacts and noise levels. Analysts shall interpret data that most clearly represents the sample and try to achieve better resolution if a sample has peak heights that are too high (resulting in artifacts, increased instrument noise, and pull-up) or too low (resulting in allelic drop out and loss of data).

2.8.19.8 Only the injection(s) used for interpretation need to be printed for the case record. However, other injection runs shall be noted in the case record and all data shall be saved under each associated laboratory case number and request folder located in the analyst's folder on the server. If individual samples in a case use different injection parameters, it shall be noted in the case record which injection was used for interpretation for each sample.

2.8.20 Archiving Applied Biosystems 3130 and GeneMapper® *ID* Projects

2.8.20.1 The 3130 run folders containing all sample files for a case as well as the GeneMapper® *ID-X* project files shall be saved under each associated laboratory case number and request folder located in the analyst's folder on the server and deleted from the hard drive of the instrument and/or analysis computer. The data stored on the server shall be routinely backed up to ensure security of data

2.8.20.2 A copy of all electropherograms used in interpretation as well as a print-out of the plate record shall be placed in the case record.

2.8.20.3 All processed plate records shall be deleted from the Data Collection Software database weekly. Run folders containing the sample files and the GeneMapper® *ID-X* projects shall be deleted on or after the 15th of each month on the instrument computers. It shall be each analyst's responsibility to ensure that all data is backed up prior to the 15th of the month.

2.8.21 Interpretation Guidelines For PowerPlex® 16 HS

2.8.21.1 Scope

2.8.21.1.1 The following interpretation guidelines shall aid in peak/true allele determinations and profile interpretations.

2.8.21.1.2 It shall be at the analyst's discretion, based on experience and training, as to which peaks are suitable for interpretation.

2.8.21.1.3 If an analyst has determined that a peak that has been labeled by the GeneMapper® *ID-X* software is not a true allele peak, the analyst can rename the allele call label in GeneMapper® *ID*-X or manually mark the change on the printed electropherogram.

2.8.21.2 Preliminary Evaluation of Allele Peaks

2.8.21.2.1 The analytical and stochastic threshold shall be determined during validation. The minimum peak height threshold is established at 50 relative fluorescent units (RFU) for GeneMapper® *ID-X* software analysis. The analytical threshold for data interpretation is 50 RFU. The stochastic threshold for data interpretation is 200 RFU.

2.8.21.2.2 Peaks below 50 RFU shall not be interpreted or marked on the STR summary sheet.

2.8.21.2.3 Interpretation of peaks with RFU from 50 to 200 RFU is a qualitative assessment and is based on the data and case information. An allele at these RFU can be used for interpretation purposes; however, it shall be interpreted with care. A peak in this range is an indication of possible allelic dropout at a locus. It will be reported in brackets (ex. 9,11>12[10]) on the Single Source STR Summary Sheet and on the mixture interpretation worksheet.)

2.8.21.2.4 Peaks above 200 RFU shall be used for interpretation.

2.8.21.2.5 Peak heights of analyzed samples should not exceed 7,000 RFU. Use of data with a single peak >7,000 RFU may be used at the discretion of the analyst, more than one peak >7,000 RFU may be allowed with the approval of a Supervisor and notification to the Technical Leader documented in the case record.

2.8.21.2.5.1 Profiles with peaks over 7,000 RFU should be interpreted with caution, particularly regarding quantitative aspects of interpretation such as stutter, peak height ratio, and mixture ratio assessments.

2.8.21.2.6 An analyst is required to visually confirm that all allelic ladders used for allele designation performed correctly.

2.8.21.2.7 Stutter peaks are artifacts of the amplification process. These peaks will typically be observed in the n-4 position of major peaks for tetranucleotide repeat loci or in the n-5 position of major peaks for the pentanucleotide repeat loci.

2.8.21.2.7.1 The peak heights of stutter peaks will be less intense than that of the major peak. The average observed percent stutter for each locus is listed in the table below. The mean stutter value for each locus is used as the stutter cut-off value in the marker stutter file

"PowerPlex16HS_ISPv2_IDX1.2.0_Panels.txt" for GeneMapper® *ID*-X analysis. Therefore, any peaks in the n-4 (for tetranucleotide repeats) or n-5 (for pentanucleotide repeats) stutter positions that are below these values when compared to the major peak will be automatically filtered out by the software and will not be labeled. However, actual stutter values may vary slightly. The table also lists the mean + 3 standard deviations (SD) value (99.7% confidence level) of stutter observed in validation. This column may be used as a guideline to the analyst for determining stutter peaks that were not filtered out by the software. It is the analyst's discretion to determine which allele calls may be labeled as stutter. For samples which have been over-loaded, the percent stutter calculation will not be accurate due to the saturation effect of the major peak.

2.8.21.2.7.2 Stutter peaks have also been documented at the n-8, n+4 or n+5 positions. These peaks will also have significantly less intense signal than the major peak. The interpretation of these peaks shall be at the discretion of the analyst based on their training and experience.

LOCUS	MEAN % STUTTER	MEAN + 3SD
D3	9.37	13.98
TH01	2.43	5.17
D21	8.83	13.24
D18	7.69	14.82
Penta E	2.96	7.91
D5	6.76	10.89
D13	6.62	13.40
D7	5.47	11.61
D16	7.29	12.08
CSF	5.92	9.80
Penta D	1.58	4.06
vWA	8.47	15.83
D8	6.45	10.34
TPOX	2.96	6.26
FGA	8.56	14.27

2.8.21.2.8 Artifacts have been observed and documented utilizing the PowerPlex® 16 HS amplification kit. The intensity of these peaks is directly related to signal intensity; therefore reducing the signal intensity below 3000 RFU should eliminate the appearance of these types of artifacts. If an analyst renames

the allele call of any artifact in GeneMapper® *ID-X* , it shall be labeled appropriately. Examples of documented artifacts are listed below. Several of the listed artifacts are believed to be the result of secondary structure within the DNA molecule. This secondary structure causes differential migration; thus the artifact location listed is an approximate value.

2.8.21.2.8.1 vWA – n-10 bp, n-11 bp, n-18 bp, n-2 bp, n+2 bp, n+4 bp

2.8.21.2.8.2 D5 – n-9 bp, a floating artifact from 114 to 116 bp and 137 to 139 bp

2.8.21.2.8.3 D21 – n-2 bp, n+2 bp, n+4 bp

2.8.21.2.8.4 TPOX – n-21bp

2.8.21.2.8.5 TH01 – n-13 bp

2.8.21.2.8.6 Penta D - n-9 bp

2.8.21.2.8.7 Amelogenin - 98 bp, 100 bp, 102 to 103 bp

2.8.21.2.8.8 Other low level artifacts are seen in the green and yellow channels. These may be located 8 to 26 bases smaller than TPOX alleles and 6 to 21 bases smaller than vWA alleles. These are more likely to be seen when peak heights are greater than 2000 rfu and when the sample is homozygous at that locus.

2.8.21.2.9 Pull-up or bleed through peaks can occur if signal intensity of sample or ILS peaks is too high or if a new spectral calibration needs to be run. Any pull-up peaks called as alleles by the GeneMapper® *ID-X* software should be labeled on the electropherogram as pull-up. The sample should be re-run if a pull-up peak interferes with the analyst's ability to evaluate the profile based on their experience and training.

2.8.21.2.10 Spikes are peaks that generally appear in all colors and are sharper than regular peaks; however, they can occur predominantly in one color. Spikes are a natural consequence of capillary electrophoresis and can be caused by dust present in the system as well as urea crystals in the system. It is essential that the instrumentation be maintained and cleaned regularly to minimize the appearance of spikes. All spikes called as alleles by the GeneMapper® *ID-X* software should be clearly labeled as spikes on the electropherogram printout. A sample should be re-injected when a spike interferes with the analyst's ability to evaluate the profile based on their experience and training.

2.8.21.2.11 Rare variants (microvariants) have been described in the literature. These peaks will have a similar intensity to the other major peak for that locus but will not line up with the

allelic ladder or have a bin in the GeneMapper® *ID-X* PowerPlex® 16 HS panels and bins settings.

2.8.21.2.11.1 Alleles one, two or three nucleotides shorter than the common four base repeat alleles (or four nucleotides shorter in the case of five base repeat alleles) which are located between two alleles on the ladder shall be described as the short repeat followed by the number of base pairs it is larger (a 0.1, 0.2, 0.3, or 0.4 in the case of a pentanucleotide repeat). Therefore, if a peak is 1 base pair larger than the 5 allele it shall be designated as 5.1. The precision of sizing at a 99.7% confidence level is less than 0.25 bp which is precise enough to be confident in the sizing of microvariants. A microvariant 4 base pairs larger than an allele (or 5 base pairs for a pentanucleotide) on the ladder may be designated with the full repeat number (A peak 4 base pairs larger than the 5 allele could be designated a 6; 5 base pairs larger a 6.1).

2.8.21.2.11.2 Alleles which are located outside the range of the ladder or bin set (above or below) shall be described as "<" or ">" the largest or smallest allele for that locus with a set of ( ) placed around the off ladder allele. For example, if a band is located above the largest allele for the D16 locus, it would be designated as "(>15)". This should be clear when used and can be verified with a locus review of the electropherogram.

2.8.21.2.11.3 Any allele peak that is not present in the allelic ladder and does not have an associated "bin" in the GeneMapper® *ID-X* analysis software, shall be called "OL" by the software. The analyst can rename the allele in the software.

2.8.21.3 Evaluation of Controls

2.8.21.3.1 Failed controls require the documented notification to the Technical leader with appropriate documentation in the case record.

2.8.21.3.2 The appearance of pull-up or known artifact peaks does not render the following controls inconclusive.

2.8.21.3.3 **Reagent Blank**:

2.8.21.3.3.1 The purpose of the reagent blank is to determine if the reagents used to extract the associated samples were contaminated by human DNA. Therefore no signal should be detected in this sample well other than the internal lane standard. If a signal is detected in the reagent blank, all results of samples associated with that reagent blank shall be considered inconclusive.

2.8.21.3.3.2 A reagent blank with peaks below 50 RFU shall not prevent associated samples from being interpreted.

2.8.21.3.3.3 A reagent blank with peaks of 50 RFU and above shall be considered a failed negative control. All associated samples shall be inconclusive. All the samples shall be repeated when appropriate.

2.8.21.3.4 **Positive Control**:

2.8.21.3.4.1 The 9947A or 2800M DNA is used as a positive control to demonstrate that the kit is performing properly. If the expected alleles are not detected in the positive control well, then the test is considered inconclusive. Due to the chromosome content of the 9947A cell line, peak height imbalance may be observed when this DNA is used as the positive control.

STR Locus	9947A	2800M
D3S1358	14,15	17,18
TH01	8,9.3	6,9.3
D21S11	30,30	29,31.2
D18S51	15,19	16,18
Penta E	12,13	7,14
D5S818	11,11	12,12
D13S317	11,11	9,11
D7S820	10,11	8,11
D16S539	11,12	9,13
CSF1PO	10,12	12,12
Penta D	12,12	12,13
Amelogenin	X,X	XY
vWA	17,18	16,19
D8S1179	13,13	14,15
TPOX	8,8	11,11
FGA	23,24	20,23

2.8.21.3.5 **Amplification Blank**

2.8.21.3.5.1 The purpose of the amplification blank is to determine if human DNA contaminated the samples at the amplification step. Because no template DNA was placed in the reaction tube, the sample well should be blank except for the internal lane standard peaks. If amplified product is detected in the amplification blank well, the test is considered inconclusive.

2.8.21.3.5.2 An amplification blank with peaks below 50 RFU shall not prevent associated samples from being interpreted.

2.8.21.3.5.3 An amplification blank with peaks of 50 RFU and above shall be considered a failed negative control. All

associated samples shall be inconclusive. All the samples shall be repeated when appropriate.

2.8.21.4 General Interpretation Considerations

2.8.21.4.1 The "phenotype" of each profile shall be recorded. A homozygous locus shall be notated by a single allele.

2.8.21.4.2 A sample with a partial profile that has interpretable peaks at one or more loci can be reported even though no peaks are detected in the remaining loci.

2.8.21.4.3 During interpretation, the analyst and technical reviewer shall each compare all unknown profiles to available staff profiles to ensure that samples have not been contaminated. All instances of profiles consistent with a staff member shall be reported to the Technical Leader and documented in the case record.

2.8.21.4.4 Test results from a question sample may not be suitable for comparison to a known standard and may have the following conclusions reported:

2.8.21.4.4.1 No results: No peaks were detected in the electropherogram.

2.8.21.4.4.2 Inconclusive: Peaks were observed at one or more loci; however no conclusive results can be drawn from them.

2.8.21.4.5 Test results from a question sample that are suitable for comparison to a known standard may have the following conclusions reported:

2.8.21.4.5.1 Consistent/Cannot Be Excluded: The profile obtained from the question stain had no discrepant alleles as compared to the profile of the known standard. (Allelic drop-out may occur in low concentrations or mixtures.)

2.8.21.4.5.2 Not consistent/Excluded: The profile obtained from the question stain had discrepant alleles as compared to the profile of the known standard, i.e. was not the same.

2.8.21.4.6 If conclusive results are obtained from a sample, appropriate, CODIS eligible profiles which do not match the victim shall be entered into the Indiana DNA Database for searching.

2.8.21.5 Single Contributor DNA Profile Interpretation

2.8.21.5.1 Generally, a single source profile should contain no more than two alleles at all loci examined. However, three-peak allele patterns have been reported for single-source stains, but these instances are rare. A profile where only one locus demonstrates three alleles could be an indication of:

2.8.21.5.1.1 A mixture where the minor contributor is not at levels allowing interpretation;

2.8.21.5.1.2 Extraneous DNA;

2.8.21.5.1.3 A tri-allelic pattern. If a tri-allelic pattern is indicated, it should be confirmed by concordance to at least one additional sample contained within the case and/or re-amplification.

2.8.21.5.2 In situations 1 and 2 above, report wording to the affect that one lone allele was detected and no conclusion can be reached is appropriate. In situation 3, no mention is required in the report.

2.8.21.5.3 For single source profiles where three alleles are detected at a single locus, the ">" shall be used to indicate if the peak height ratio of the two smallest alleles is less than 0.33 on the Single Source STR Summary Sheet.

2.8.21.5.4 For single source profiles, the peak height ratio (the smallest peak divided by the largest peak) of heterozygous individuals at a locus should be within 0.65.

2.8.21.5.4.1 Samples may display peak height ratios less than expected when the sample is of poor quality or the amplification target is below 0.5 ng of template DNA.

2.8.21.6 Single Contributor Statistical Analysis (if necessary)

2.8.21.6.1 The Random Match Probability (RMP) statistic will be calculated for single source samples, when appropriate.

2.8.21.6.2 A locus that demonstrates only a single allele that is between 50 RFU and 201 RFU or that indicates allelic drop-out shall not be used in the statistical calculation.

2.8.21.6.3 A single heterozygous locus with one or both alleles between 50 RFU and 201 RFU may be included in a RMP calculation. Supervisor approval is required to use more than one locus.

2.8.21.6.4 The Single Source STR Summary sheet shall be marked for loci of the question sample that are determined to be suitable for the RMP calculation, before comparison to associated standards. A check mark shall be placed in the appropriate box to indicate a locus where no drop-out is suspected.

2.8.21.6.5 When a profile is consistent with a standard and the most common frequency exceeds 330 billion for each population group an identity statement shall be used for that item.

2.8.21.7 Multiple Contributor DNA Profile Interpretation

2.8.21.7.1 Assumptions used in the interpretation of mixtures shall be documented on the mixture interpretation worksheet.

2.8.21.7.2 Analysts shall consider the additive effects of allele sharing during interpretation.

2.8.21.7.3 The flow of mixture interpretation generally follows the steps listed below (Clayton, et al., 1998):

2.8.21.7.3.1 Identify the presence of a mixture.

2.8.21.7.3.2 Designate the allele peaks.

2.8.21.7.3.3 Identify the minimum number of contributors.

2.8.21.7.3.4 Estimate the relative ratio of the individual contributors to the mixture.

2.8.21.7.3.5 Consider all genotype combinations.

2.8.21.7.3.6 Compare reference samples.

2.8.21.7.3.7 Statistical analysis (if necessary).

2.8.21.7.4 **Identify the Presence of a Mixture**

2.8.21.7.4.1 A profile is defined as a mixture between two or more individuals when two or more loci demonstrate three or more alleles.

2.8.21.7.4.1.1 A profile where only one locus demonstrates three alleles could be an indication of:

2.8.21.7.4.1.1.1 A mixture where the minor contributor is not at levels allowing interpretation;

2.8.21.7.4.1.1.2 Extraneous DNA;

2.8.21.7.4.1.1.3 A tri-allelic pattern. If a tri-allelic pattern is indicated, it should be confirmed by concordance to at least one additional sample contained within the case and/or re-amplification.

2.8.21.7.4.1.2 A peak height ratio less than 0.65 at a locus may indicate a mixed profile and that allele sharing may be occurring.

2.8.21.7.4.1.3 Peaks in stutter position that exceed the expected stutter percentage may indicate the presence of a mixture.

2.8.21.7.4.1.4 All loci shall be considered when determining the presence of a mixture.

2.8.21.7.5 **Designation of allele peaks**

2.8.21.7.5.1 Alleles are designated as previously described in 2.8.21.2.

2.8.21.7.5.1.1 A peak in stutter position should be considered a possible allele peak if it exceeds the established stutter percentage (mean + 3SD) or if its peak height is equal to or greater than the peak height of minor contributor alleles.

2.8.21.7.5.1.1.1 Stutter peaks shall be evaluated per locus.

2.8.21.7.5.1.1.2 For loci demonstrating peaks below the stochastic threshold, stutter need not be evaluated.

2.8.21.7.5.1.1.3 If no independent minor contributor peaks are observed at a locus, peaks within expected stutter range should not be considered.

2.8.21.7.5.1.1.4 The assumed number of contributors may be used to eliminate peaks in stutter position as possible alleles.

2.8.21.7.5.1.1.5 Peaks in stutter position that have been filtered out by GeneMapper® *ID-X* should be considered for interpretation. The RFU value and allele call of filtered stutter peaks shall be manually recorded on the printed electropherogram if being considered for interpretation.

2.8.21.7.6 **Identify the minimum number of contributors**

2.8.21.7.6.1 The number of contributors in the sample shall be determined using peaks above analytical threshold.

2.8.21.7.6.2 The minimum number of contributors should be determined using the locus demonstrating the greatest number of alleles.

2.8.21.7.6.3 A profile in which a locus demonstrates more than 6 alleles shall be considered too complex for interpretation. An exception may be made with Technical Leader approval and review of the final interpretation documented in the case record.

2.8.21.7.7 **Assumed Two Person Mixture Interpretation**

2.8.21.7.7.1 A mixed DNA profile can be classified as an indistinguishable mixture, a mixture demonstrating a major contributor, and/or a mixture that demonstrates the potential for allelic drop-out.

2.8.21.7.7.2 The average mixture ratio shall be calculated for samples indicating a possible major profile or for intimate samples in which a foreign profile may be deduced.

2.8.21.7.7.2.1 Calculate the average mixture ratio using all loci demonstrating four alleles.

2.8.21.7.7.2.2 The mixture ratio shall be calculated by dividing the sum of the peak heights of the two largest alleles by the sum of the peak heights of the two smallest alleles.

2.8.21.7.7.2.3 The mixture ratio shall be reported to three significant figures, with no rounding.

2.8.21.7.7.3 Determination of a major profile

2.8.21.7.7.3.1 The average mixture ratio shall be at least 3:1.

2.8.21.7.7.3.2 At least eight loci are required for a major profile. Exceptions require approval of a supervisor with notification to the Technical Leader documented in the case record.

2.8.21.7.7.3.2.1 Loci with 4 alleles:

1. If the calculated mixture ratio for the individual locus is ≥3:1 and the peak height ratio of the two highest alleles is ≥0.65, the two highest alleles may be called as the major component.
2. If the calculated mixture ratio for the individual locus is <3:1, the locus is reported as having no major profile.

2.8.21.7.7.3.2.2 Loci with 3 alleles:

1. Determine if the peak height ratio of the two highest alleles is ≥ 0.65. (if yes go to #2, if no go to #4)
2. Determine if the peak height ratio of the third allele is ≥ 0.33 ratio of either of the other alleles. (if yes go to #5, if no go to #3)
3. Report the two highest alleles as the major profile with the third allele as a minor on the mixture interpretation worksheet.
4. Do the two shorter alleles add up to ≥ 0.33 of the larger allele? (if yes go to #5, if no go to #6)
5. Report the locus as having no major.
6. Report the higher allele as a homozygote major type and other alleles as the minor type on the mixture interpretation worksheet.

2.8.21.7.7.3.2.3 Loci with 2 alleles:

1. Determine the peak height ratio of the two alleles and proceed as follows:

2. If the ratio is ≤0.14 then report the locus as having a major with tallest peak being the major on the mixture interpretation worksheet.
3. If the ratio is >0.14 and <0.65 then report the locus as having no major.
4. If the ratio is ≥ 0.65 then report both alleles as a major on the mixture interpretation worksheet.

2.8.21.7.7.3.2.4 Loci with 1 allele:

1. Evaluate the profile overall and determine that there are no conditions that exist that may result in allelic drop-out at this locus. If allelic drop-out of the major profile is not suspected, a major profile may be reported on the mixture interpretation worksheet.

2.8.21.7.7.4 Mixture deconvolution using a known contributor's standard.

2.8.21.7.7.4.1 Mixture deconvolution using a known contributor profile shall only be performed on intimate sample profiles.

2.8.21.7.7.4.2 The known contributor profile may be used to deduce the obligate alleles of the second contributor.

2.8.21.7.7.4.3 Variation beyond a 0.65 peak height ratio may be an indication that allele sharing may be occurring.

2.8.21.7.7.4.4 At loci where only one obligate allele can be determined, a "#" will indicate on the Mixture Worksheet if a second obligate allele is in question.

2.8.21.7.7.4.5 A single obligate foreign allele between 50 RFU and 201 RFU cannot be evaluated confidently due to the possibility of allelic dropout. Therefore, that locus shall not be included in the reported deduced profile. If there are two independent obligate foreign alleles and one or both fall between 50 RFU and 201 RFU, the locus may be included in the reported deduced profile.

2.8.21.7.7.4.6 A minimum of eight loci with assumed complete representation of the deduced profile are required to make a conclusion.

2.8.21.7.8 **Greater Than Two Person Mixture Interpretation**

2.8.21.7.8.1 A greater than two person mixed DNA profile can be classified as an indistinguishable mixture, a mixture demonstrating major contributors as part of the mixture, and/or a mixture that demonstrates the potential for allelic drop-out.

2.8.21.7.8.2 Due to possible allele sharing, a single source major profile shall only be determined with Technical Leader approval documented in the case record.

2.8.21.7.8.3 A two person mixed major profile may be determined.

2.8.21.7.8.3.1 The peak height ratio between the highest allele of the minor contributor and the lowest allele of the major contributors shall be <0.33.

2.8.21.7.8.3.2 A deduced profile may be determined from an assumed two person mixed major profile in an intimate sample using guidelines described in 2.8.21.7.7.4.

2.8.21.7.9 **Multiple Contributor Statistical Analysis (if necessary)**

2.8.21.7.9.1 The Combined Probability of Inclusion (CPI) statistical calculation shall be done when a conclusion is drawn that includes a possible contributor to a mixture and the evidence is meaningful.

2.8.21.7.9.1.1 Mixtures where some of the alleles are not attributable to the owner of the item or to any standards provided shall have statistics calculated and reported out when significant to the case.

2.8.21.7.9.2 If a single source major can be clearly distinguished at a minimum of eight loci, the major profile can be calculated with the RMP. The mixed DNA profile can be calculated using an unrestricted CPI.

2.8.21.7.9.3 If a mixed major profile can be clearly distinguished at a minimum of eight loci, a restricted CPI calculation can be calculated that includes the alleles designated to be in the major. The entire mixed DNA profile can also be calculated using an unrestricted CPI.

2.8.21.7.9.4 If a deduced profile is determined from an intimate sample, the deduced profile can be calculated as a single source (RMP).

2.8.21.7.9.4.1 At least eight loci must be marked with full representation of the deduced profile to be used in the RMP calculation.

2.8.21.7.9.5 Unrestricted CPI calculations shall include all alleles present at each locus for the loci determined suitable for the statistical calculation. Restricted CPI calculations shall include all alleles determined to be present in the mixed major DNA profile.

2.8.21.7.9.5.1 All possible peaks in stutter position that cannot be confidently eliminated as stutter peaks shall be included in the mixture statistical calculation.

2.8.21.7.9.5.2 Any loci demonstrating possible allelic drop-out shall not be included in calculation of the CPI.

2.8.21.7.9.5.3 All alleles at a locus shall be above 200 RFU for the locus to be used in an unrestricted CPI calculation.

2.8.21.7.9.5.4 All alleles used in the restricted CPI calculation for a mixed major profile shall be above 200 RFU.

2.8.21.7.9.5.5 Possible allelic drop-out may be demonstrated by alleles in [ ] or by peaks visible below the 50 RFU threshold. An analyst may use their discretion to **not** mark any locus when they feel the potential for drop-out is indicated by a general low quality/low RFU profile.

2.8.21.7.9.6 If no loci in a profile are eligible for the CPE/CPI calculation, the results shall be reported as inconclusive.

2.8.21.7.9.7 The mixture interpretation worksheet shall be marked for loci of the question sample that are determined to be suitable for the CPI calculation, before comparison to associated standards. A check mark shall be placed in the appropriate box to indicate a locus where no drop-out is suspected.

2.8.21.7.9.7.1 If a locus has been correctly marked with a check mark as not demonstrating the potential for drop-out and it is the only locus excluding an individual as a contributor to the mixture, the individual is excluded.

2.8.21.7.9.7.2 If a locus has been marked with a checkmark as not demonstrating the potential for drop-out and subsequently during comparison with standards allelic drop-out is identified, the sample will be inconclusive. Therefore, there will be no conclusion as to the inclusion/exclusion of any individuals. Further testing of the sample (extraction and/or amplification) may be attempted to determine if further information may

be obtained to allow a conclusion as to an inclusion/exclusion of the individual in question.

2.8.21.7.10 **General Rules for Statistical Calculations** (See Stats reference sheet)

2.8.21.7.10.1 Statistics may be calculated using "IndyStats" or by hand calculation. Statistics calculated by "IndyStats" shall automatically be rounded to the appropriate number of significant figures.

2.8.21.7.10.2 Statistics shall be calculated for all meaningful profiles. Examples of profiles that do not need statistics calculated would include single source profiles of unknown individuals or the profile of the owner on intimate samples (must calculate when the identity statement is used in reporting out these samples).

2.8.21.7.10.3 If no loci are eligible for a statistical calculation, no conclusions shall be drawn from that sample and it shall be reported as failed to demonstrate conclusive results.

2.9 Records

2.9.1 The appropriate worksheets as contained in the Worksheet Manual or the equivalent workbooks shall be used to record all procedures.

2.9.2 All data sheets, notes, photographs, and other information generated from the laboratory examination shall be kept in the case record.

2.9.3 The technical review of the case record shall be recorded on the technical review worksheet.

2.9.4 Electronic records shall be retained as indicated in Appendix 5.

2.10 Interpretations of Results

2.10.1 Interpretations guidelines are located within the Procedures section 2.8.21.

2.11 Report Writing

2.11.1 General rules

2.11.1.1 Serology results shall be reported prior to any DNA results. DNA results shall be preceded by the appropriate introductory statement. No headers shall be used between sections of the report.

2.11.1.2 Item numbers shall contain all leading zeros.

2.11.1.3 The retention statement should be at the end of serology results when present. If no serology results are being reported, the retention statement shall be the last statement in the report.

2.11.1.4 Full names of victim and suspect shall be used throughout the entire report.

2.11.1.5 When a person or a group of people are excluded from a sample, they do not have to be mentioned at every sample from which they are excluded.

They can be added to a paragraph at the end of the Results/Opinions/Interpretations area stating that the standards were used for comparison purposes.

2.11.1.6 A unique unknown individual may be indicated in a single source sample, major profile in a mixed sample, or deduced profile from an intimate sample.

2.11.1.6.1 A unique unknown individual can be reported as being included/excluded from other samples in the case record at the analyst's discretion.

2.11.1.7 Specific genotypes shall not be reported.

2.11.1.8 Items should be grouped together by results, and paragraphs arranged in order of significance.

2.11.1.9 When differential extractions are employed, the terminology "sperm {cell} fraction" and "non-sperm {cell} fraction" shall be used in the report.

2.11.1.10 When reporting a mixture, the assumed number of contributors shall be stated.

2.11.1.11 When two or more samples are combined at any point in the analysis process, the report shall refer to the samples as "combined". The analyst's notes shall clearly describe which samples and at which stage of the process the samples were combined.

2.11.1.12 When a cross-case comparison is performed, the item #, Indiana State Police Laboratory case #, and Agency case # shall be used to identify the appropriate item.

2.11.1.12.1 If the comparison is performed to a different agency's case implicating an individual, additional identifiers for the individual must be included whenever possible. (i.e. birth date or Department of Corrections number).

2.11.1.13 The appropriate CODIS statement shall be included, prior to any reported statistics, for every report detailing a new DNA profile.

2.11.1.14 Report wording for a CODIS hit is available in the Biology Databasing Test Method.

2.11.1.15 Report wording for outsourced cases is available in the Outsource Manual.

2.11.1.16 Report wording may be altered with the approval of a Biology Unit Supervisor.

2.11.2 Statistics rules

2.11.2.1 Only the most complete statistical evaluation of the profile from an item or location with multiple profiles from one individual is required to be reported.

2.11.2.2 Statistical evaluations of samples matching the person from whom they were collected are not required to be reported.

2.11.2.3 If multiple samples have the same statistical calculation and it is necessary to report out one sample, all samples shall be listed in the statistical paragraph.

2.11.2.4 When reporting out frequency, it will be to the nearest whole person or 2 significant figures. (ex. 1 in 6.8 should be reported as 1 in 6 or 10 in 68)

2.11.3 Wording Of DNA Results/Opinions/Interpretations: (required) {options} [example]

WITHDRAWAL STATEMENT

The request for {DNA} analysis was withdrawn by John Smith, [Anywhere Police Department], on [date].

INTRODUCTORY STATEMENTS:

DNA Profiles Generated Using PowerPlex ® 16 HS: (Any additional loci analyzed shall also be listed)

In the DNA analysis detailed below, the following STR loci were analyzed by Polymerase Chain Reaction (PCR): D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, vWA, D8S1179, TPOX, FGA, and Amelogenin.

No Samples Amplified:

Extraction and quantification were performed in the DNA analysis of the sample{s} detailed below.

SINGLE SOURCE STATEMENTS:

The DNA profile obtained from X (item #) is {not} consistent with John Doe (item #).

The DNA profile obtained from X (item #) matches the DNA profile of John Doe (item #). In the absence of an identical twin, John Doe is the source of the DNA to a reasonable degree of scientific certainty.

Detection of One Or Two Additional Alleles:

X (item #) demonstrate a DNA profile consistent with Jane Doe (item #); however, {one/two} additional allele{s} {was/were} detected. No conclusion can be drawn from the additional allele{s}.

The DNA profile obtained from X (item #) matches the DNA profile of John Doe (item #); however, {one/two} additional allele{s} {was/were} detected. In the absence of an identical twin, John Doe is the source of the DNA to a reasonable degree of scientific certainty. No conclusion can be drawn from the additional allele{s}.

MIXTURE STATEMENTS:

Indistinguishable Mixture:

The DNA profile obtained from X (item #) demonstrated the presence of a mixture of at least # individuals from which John Doe (item #) and Jane Doe (item #) cannot be excluded as possible contributors.

Distinguishable Single Source Major Profile:

The DNA {profile/result} obtained from X (item #) demonstrated the presence of a mixture of at least # individuals. One distinct profile was detected that is consistent with John Doe (item #). {Jane Doe (item #) can{not} be excluded as a possible contributor of the additional alleles/No conclusion can be drawn from the additional alleles.}

The DNA {profile/result} obtained from X (item #) demonstrated the presence of a mixture of at least # individuals with a major DNA profile. In the absence of an identical twin, Jane Doe (item #) is the source of the major DNA profile to a reasonable degree of scientific certainty. {John Doe (item #) can{not} be excluded as a possible contributor of the additional alleles/No conclusion can be drawn from the additional alleles.}

Distinguishable Mixed Major Profile:

The DNA {profile/result} obtained from X (item #) demonstrated the presence of a mixture of a least # individuals with a major DNA profile of at least two individuals. Jane Doe (item #) and John Doe (item #) cannot be excluded as possible contributors of the major profile. {Joe Smith (item #) can{not} be excluded as a possible contributor of the additional alleles/No conclusion can be drawn from the additional alleles.}

Deduced Profile Determined From An Intimate Sample:

An assumed two person DNA mixture was obtained from X (item #). Assuming Jane Doe (item #) as a contributor, a deduced profile was developed that is consistent with John Doe (item #).

An assumed two person DNA mixture was obtained from X (item #). Assuming Jane Doe (item #) as a contributor, a deduced profile was developed that matches John Doe (item #). In the absence of an identical twin, John Doe is the source of the deduced DNA profile to a reasonable degree of scientific certainty.

Deduced Profile Determined From The Mixed Major Portion Of An Intimate Sample:

The DNA {profile/result} obtained from X (item #) demonstrated the presence of a mixture of at least # individuals with an assumed two person major DNA profile. Assuming Jane Doe (item #) as a contributor to the major DNA profile, a deduced profile was developed from the major profile that is consistent with John Doe (item #). {Joe Smith (item #) can{not} be excluded as a possible contributor of the additional alleles/No conclusion can be drawn from the additional alleles.}

The DNA {profile/result} obtained from X (item #) demonstrated the presence of a mixture of at least # individuals with an assumed two person major DNA profile. Assuming Jane Doe (item #) as a contributor to the major DNA profile, a deduced

profile was developed from the major profile that matches John Doe (item #). In the absence of an identical twin, John Doe is the source of the deduced DNA profile to a reasonable degree of scientific certainty. {Joe Smith (item #) can{not} be excluded as a possible contributor of the additional alleles/No conclusion can be drawn from the additional alleles.}

SAMPLES UNSUITBALE FOR COMPARISON:

X (item #) failed to demonstrate a DNA profile.

The DNA profile obtained from X (item #) failed to demonstrate conclusive results.

Due to the complexity of the mixture identified on X (item #), comparison to known samples would not provide strong statistical evidence.

SAMPLES NOT AMPLIFIED:

Insufficient Human DNA:
X (item #) failed to demonstrate a sufficient quantity of DNA for further analysis.

Insufficient Male DNA:
X (item #) failed to demonstrate a sufficient quantity of male DNA for autosomal STR analysis.

Standards:

When Introductory Statement Listing Loci Analyzed Was Used:
The DNA standard of John Doe (item #) was extracted and quantified; however, no additional DNA analysis was performed due to a lack of evidence for comparison.

When No Amplification Introductory Statement Was Used:
No further DNA analysis was performed on the DNA standard of John Doe (item #) due to a lack of evidence for comparison.

STANDARD DEVELOPED FOR COMPARISON:

The DNA profile obtained from the standard of John Doe (item #) {was/will be} used for comparison purposes.

SAMPLE/SUBITEM NOT EXAMINED:

No DNA analysis was performed on {# additional samples within} item # at this time.

Due to limited sample size, no DNA analysis was performed on X (item #) at this time.

No DNA analysis was performed on X (item #) at this time. If further DNA analysis is desired, permission to consume the sample is required. Please contact the reporting analyst.

INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS

REQUESTS FOR STANDARDS:

Standard For Comparison:

Additional comparisons will be made upon submission of an appropriate DNA standard (such as a blood standard in a purple top tube or an oral swab standard) from (victim's name) and/or {any} suspect{s}.

Standard For Y-STR Analysis:

Upon submission of an appropriate DNA standard (such as a blood standard in a purple top tube or an oral swab standard) from any suspects, this case {may/will} be evaluated for Y-STR analysis.

SAMPLES FORWARDED FOR ADDITIONAL ANALYSIS:

Y-STR Evaluation Pending:

This case will be evaluated for possible Y-STR analysis, any results from additional testing will be provided in a separate report.

Criminal Paternity/Kinship Analysis Pending:

When completed, {Paternity/Kinship} analysis of the DNA profiles determined for John Doe (item #), Jane Doe (item #), and Baby Doe (item #) will be provided in a separate report.

CONFIRMATION OF SPERMATOZOA DURING DIFFERENTIAL EXTRACTION:

DNA testing confirmed the presence of {semen/seminal material} on the sperm {cell} fraction of X (item #).

IDENTIFICATION OF CONTAMINATION FROM LABORATORY PERSONNEL:

No DNA profile of apparent value was identified on X (item #). The DNA profile of a {laboratory analyst/non-laboratory person} was identified on item #.

The DNA profile obtained from X (item #) demonstrated the presence of a mixture of at least # individuals with a major DNA profile. The major DNA profile is consistent with Jane Doe (item #). The DNA profile of a {laboratory analyst/non-laboratory person} cannot be excluded as a possible contributor to the mixture.

IDENTIFICATION OF IDENTICAL TWINS:

At The Start Of Results Section:

The DNA profiles obtained from Jane Doe (item #) and Susan Doe (item #) demonstrated the same allelic profile. This is typically associated with identical twins. The following conclusions reflect the comparisons with the provided standards.

Representative Results Paragraph: (Not all possible combinations are given, consultation with the Technical Leader and/or Supervisor is recommended.)

The DNA profile obtained from X (item #) matches the DNA profile of Jane Doe (item #) and/or Susan Doe (item #). Because identical twins are genetically identical, Jane Doe and/or Susan Doe are the source of the DNA to a reasonable degree of scientific certainty.

CODIS STATEMENTS:

Profile Entered:

The {appropriate portions of the} DNA profile developed from X (item #) {was/were} entered into the Indiana DNA Database and will be searched on a routine basis. In the event of a database match, information regarding the match(es) will be provided in a separate report.

Pending Standard Submission:

Upon submission of an appropriate DNA standard from (victim's name), the DNA profile obtained from X (item #) will be re-evaluated for possible entry into the Indiana DNA Database.

Pending Additional Information:

The DNA profile obtained from X (item #) is consistent with an unknown male. Additional information is required regarding X before it can be determined if the profile is eligible to be searched in the Indiana DNA Database. Please contact the reporting analyst if a database search is desired.

No Profiles Suitable:

No DNA profiles were suitable for entry into the Indiana DNA Database.

WORDING OF STATISTICAL ANALYSIS:

When Reporting A RMP Calculation:

The {deduced/major} DNA profile obtained from X (item #), which is consistent with John Doe (item #), occurs in the approximate percentages of the populations of unrelated individuals listed below:

When Reporting An Unrestricted CPI Calculation On A Mixture:

The estimates of the combined probability of inclusion (i.e. the chance of selecting an unrelated individual at random that would be included) for the DNA profile obtained from X (item #), which demonstrated the presence of a mixture from which John Doe (item #) {and Jane Smith (item #)} could not be excluded as being {a} possible contributor{s}, occur in the approximate percentages of the populations of unrelated individuals listed below:

When Reporting A Restricted CPI Calculation On A Mixed Major DNA Profile:

The estimates of the combined probability of inclusion (i.e. the chance of selecting an unrelated individual at random that would be included) for the major portion of the DNA profile obtained from X (item #), which demonstrated the presence of a mixture from which John Doe (item #) and Jane Smith (item #) could not be excluded as

being possible contributors, occur in the approximate percentages of the populations of unrelated individuals listed below:

When Statistical Analyses Are Being Reported, The Table Below Including A List of Loci Used For Calculations And Following Note Shall Be Included:

POPULATION	PERCENTAGE	1/FREQUENCY
CAUCASIAN	%	1 in #
AFRICAN AMERICAN	%	1 in #
HISPANIC	%	1 in #

Loci used in the above calculation include: (list loci)

Note: The combined probability of inclusion (CPI) is an estimate of the proportion of individuals that would be possible donors to the observed DNA mixture. The method applies the recommended concepts endorsed by the Scientific Working Group on DNA Analysis Methods (1/4/10). **[This paragraph only needs to be placed once per report involving mixtures.]**

When A Statistical Estimate Has Been Calculated But Is Not Being Reported:
The statistical estimates for the DNA profile{s} obtained from X (item #) have been calculated and are available upon request.

RETENTION STATEMENT:

All subitems created from originally submitted items will be retained by the Indiana State Police Laboratory for the possibility of future analysis.

2.12 References:

2.12.1 Federal Bureau of Investigation. Procedures for the Detection of Restriction Fragment Length Polymorphisms in Human DNA. FBI Laboratory. 1990.

2.12.2 Federal Bureau of Investigation. PCR-Based Typing Protocols. FBI Laboratory. 1994.

2.12.3 Applied Biosystems. GeneAmp® PCR System 9700 User's Manual. P/N 4331608, rev. B. 2003

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3 Forensic Relationship Comparison Methods

3.1 Scope

3.1.1 Forensic relationship comparisons may be requested as part of some criminal investigation cases. Types of relationship comparisons that can be performed include paternity, maternity, reverse paternity, sibling relationship (sibship), etc. These comparisons may establish potential relationships among individuals and/or aid in the identification of human remains and missing persons. Samples for forensic relationship testing shall be processed in accordance to the test methods outlined in the DNA methods section of this document.

3.2 Precautions/Limitations

3.2.1 Cases submitted for relationship comparisons should be evaluated by a member of the relationship comparison team before any testing is performed. It may be necessary to outsource some types of cases to a vendor laboratory for analysis. Cases shall be refused when they do not meet the requirements of forensic relationship comparison analysis.

3.3 Related Information

3.3.1 See Relationship Comparison Statistical Reference Sheet (Appendix 6)

3.4 Instruments

3.4.1 See DNA Test Methods Section 2.4

3.5 Reagents/Materials

3.5.1 See DNA Test Methods Section 2.5

3.6 Hazards/Safety

3.6.1 See DNA Test Methods Section 2.6

3.7 Reference Materials/Controls/Calibration Checks

3.7.1 See DNA Test Methods Section 2.7

3.8 Procedures/Instructions

3.8.1 See DNA Test Methods Section 2.8

3.9 Interpretations of Relationship Testing Results

3.9.1 Possible Outcomes of Relationship Comparisons

3.9.1.1 The genetic profiles from standards and samples are compared to evaluate relationship. The following conclusions may be reported:

3.9.1.2 For parentage relationship cases:

3.9.1.2.1 The genetic results strongly support the hypothesis of the alleged relationship; therefore, the alleged individual cannot be excluded from the relationship. [Combined Parentage Index (CPI) >100]

3.9.1.2.2 The genetic results do not strongly support the hypothesis of the alleged relationship nor does it indicate no relationship;

therefore, it is deemed inconclusive. An inconclusive result may also be derived if a genetic profile cannot be interpreted or is of poor quality. [100 > Combined Parentage Index (CPI) > 1]

3.9.1.2.3 The genetic results support the hypothesis of no relationship; therefore, the alleged individual can be excluded from the relationship. [Combined Parentage Index (CPI) <1]

3.9.1.3 For non-parentage relationship cases:

3.9.1.3.1 The genetic results support the hypothesis of the alleged relationship. [Combined Relationship Index (CRI) >10]

3.9.1.3.2 The genetic results do not strongly support the hypothesis of the alleged relationship nor does it indicate no relationship; therefore, it is deemed inconclusive. (10 > CRI > 0.05)

3.9.1.3.3 The genetic results support the hypothesis of no relationship. (CRI <0.05)

3.9.2 Inconsistent Profiles In Parentage Relationship Cases

3.9.2.1 If the alleged father's profile is inconsistent with the child's profile at three or more loci, then no relationship/exclusion shall be concluded.

3.9.2.2 If the alleged father's profile is inconsistent with the child's profile at fewer than three loci, then other alternatives may be evaluated for these inconsistencies. Other alternatives may include possible mutation, null allele, first degree relative, or true exclusion. If it is determined that a possible mutation could account for the inconsistencies, then the mutation shall be included into the statistical calculations.

3.9.3 Mixtures

3.9.3.1 With product of conception/fetal samples, it is necessary to have a standard from the mother. Only loci where obligate paternal alleles can be determined shall be used for statistical purposes.

3.9.3.2 With personal effects believed to belong to a missing person, the degree of the mixture shall be evaluated to determine whether conclusive results can be obtained. Additional standards may be requested to aid in interpretation.

3.10 Rules for Relationship Statistical Calculations

3.10.1 Calculations shall be performed using the most current available version of Popstats (the version shall be documented in the case record), a computer software program designed by the FBI for statistical calculations.

3.10.2 Statistical calculations shall be calculated and reported in forensic relationship comparison cases where Results/Opinions/Interpretations are being given in regards to relationship. However, statistical calculations shall not be performed in parentage cases with conclusions of no relationship/exclusion.

3.10.3 See Relationship Comparison Statistical Reference Sheet (Appendix 6) for a list of formulas for calculations.

3.10.4 If more than one standard/sample is available for comparison, the most representative standard/sample or the standard/sample where the most obligate alleles can be determined shall be used for statistical calculations.

3.10.5 Relationship statistical calculations utilize the race of the alleged individual. The calculations shall be performed with the appropriate population data using the racial information provided by the contributing agency. If racial information is unknown or unobtainable, calculations shall be performed using each population group.

3.10.6 Manual calculations performed by an analyst shall be re-calculated by a qualified technical reviewer and a qualified unit supervisor.

3.10.7 A Prior Probability (Pr) of 0.5 shall be used for all relationship calculations.

3.10.8 The Paternity Index for a locus demonstrating a possible mutation shall be calculated using the average locus mutation rate (μ) and the Probability of Exclusion (PE) determined by the obligate paternal allele.

3.10.9 All mutation rates are reported by AABB (American Association of Blood Banks), Standards for Relationship Testing Laboratories, 10th Edition, Appendix 6 (or the most current available edition).

3.11 Report Writing for Relationship Comparisons

3.11.1 General Guidelines For Report Writing

3.11.1.1 The DNA profiles shall be reported in table format in the Results/Opinions/Interpretations area of the Certificate of Analysis. The Paternity Index for each locus shall be included in the table. Combined Paternity Index and Probability of Paternity shall be reported in the table when applicable.

3.11.1.2 All reports shall include the introductory statement and retention statement listed in the DNA Methods report wording section.

3.11.1.3 Alternate report wording may be used depending on the type of relationship and calculation performed.

3.11.1.4 Terminology of Parentage, Paternity, Maternity, Relationship, Kinship, and Sibling Relationship (Sibship) may be used interchangeably where applicable.

3.11.1.5 The Combined Paternity Index and the Paternity Index shall be reported as calculated by Popstats. However, any Popstats calculation of the Combined Paternity Index with a decimal value shall be rounded down to the nearest whole number.

3.11.1.6 The Probability of Paternity shall be reported to four decimal places.

3.11.1.7 Statistical calculations shall be rounded to four significant figures for manual calculations.

3.11.1.8 Example Table for Certificate of Analysis

DNA STR Locus	Mother Item 1A	Child Item 2A	Alleged Father Item 3A Race	Paternity Index
D3S1358				
TH01				
D21S11				
D18S51				
Penta E				
D5S818				
D13S317				
D7S820				
D16S539				
CSF1PO				
Penta D				
vWA				
D8S1179				
TPOX				
FGA				

Combined Paternity Index =
Probability of Paternity = %

3.11.1.9 Alternatively, the genetic results of the Amelogenin locus can also be reported in the table on the Certificate of Analysis when the gender of an individual may be meaningful.

3.11.2 Wording Of Relationship Comparisons For Results/Opinions/Interpretations (required) {options}[example]

3.11.2.1 Standards Developed for Comparison

3.11.2.1.1 The DNA profiles developed from the standards of [John Doe] (item #) and [Jane Doe](item #) were used for comparison purposes.

3.11.2.1.2 The DNA profiles previously developed from the standards of [John Doe] (item #) and [Jane Doe] (item #) were used for comparison purposes.

3.11.2.2 A Parentage Case Where Alleged Individual Cannot Be Excluded

3.11.2.2.1 [John Doe] (item #), the alleged father, cannot be excluded as the biological father of [the child] (item #). The genetic results listed below are [CPI] times more likely if [John Doe] is the biological father of [the child] than a random, untested, unrelated man in the {Caucasian/African American/Hispanic} population. The probability of paternity (assuming a prior probability of 0.5) is [W] %.

3.11.2.3 A Parentage Case Where Alleged Individual Can Be Excluded

3.11.2.3.1 Given the lack of genetic markers that must be contributed to the child by the biological father in the genetic results listed below, [John Doe] (item #), the alleged father, can be excluded as the biological father of [the child] (item #).

3.11.2.4 A Parentage Case With Inconclusive Results

3.11.2.4.1 The genetic results listed below do not strongly favor the hypothesis of [John Doe] (item #), the alleged father, being the biological father of [the child] (item #). The Combined Paternity Index of [CPI] is low and does not meet our laboratory standards in order to establish paternity; therefore, these results are inconclusive. The genetic results are based only on the current data and additional genetic testing is recommended.

3.11.2.5 A Reverse Parentage Case Where Alleged Individuals Cannot Be Excluded

3.11.2.5.1 [Jane Doe] (item #), the alleged mother, and [John Doe] (item #), the alleged father, cannot be excluded as the biological parents of [the child] The genetic results listed below are [CPI] times more likely [Jane Doe] and [John Doe] are the biological parents of [the child] than random, untested, unrelated individuals in the {Caucasian/African American/Hispanic} population. The probability of parentage (assuming a prior probability of 0.5) is [W] %.

3.11.2.6 A Sibship Comparison With Evidence Of Relationship

3.11.2.6.1 The genetic results listed below support the conclusion that [John Doe] (item #) and [Joe Doe] (item #) are related as full siblings. These genetic results are [CRI] times more likely if [John Doe] is a full sibling of [Joe Doe] than if they are unrelated. The probability of relationship (assuming a prior probability of 0.5) is [W] %.

3.11.2.7 A Sibship Comparison With No Evidence Of Relationship

3.11.2.7.1 The genetic results listed below support the hypothesis that [John Doe] (item #) and [Joe Doe] (item #) have different biological parents over the hypothesis that [John Doe] and [Joe Doe] share biological parents. These genetic results are [CRI] times more likely if [John Doe] is unrelated to [Joe Doe] than if they are full siblings. The genetic results are based only on the current data and do not supersede any additional genetic testing.

3.11.2.8 A Sibship Comparison With Inconclusive Results

3.11.2.8.1 The genetic results listed below do not strongly support the hypothesis that [John Doe] (item #) and [Joe Doe] (item #) share the same biological parents nor does it indicate that [John Doe] and [Joe Doe] have different biological parents; therefore, it is

deemed inconclusive. The Combined Relationship Index for full siblings of [CRI] is low and does not meet the laboratory standards necessary to establish a relationship. The genetic results are based only on the current data and additional genetic testing is recommended. Please contact the reporting analyst in regards to further genetic testing.

3.11.2.9 Cases With An Observed Mutation

3.11.2.9.1 A single genetic inconsistency between the child and the alleged father was observed at (Locus). This has been incorporated into the calculation as a mutation.

3.11.2.10 Cases With An Observed Mixture

3.11.2.10.1 A mixture was observed in the sample of [John Doe] (item #). The following loci were not suitable for statistical calculations as the obligate paternal allele was not able to be determined: (list loci).

3.11.2.11 Cases with Multiple Evidentiary or Standard Samples

3.11.2.11.1 DNA profiles were developed for [two] additional samples of X (item #); however, these profiles were not used for statistical purposes.

3.11.2.12 When A Statistical Calculation Is Being Reported

3.11.2.12.1 Statistical calculations were performed based on the recommendations of the AABB (American Association of Blood Banks).

3.12 Definitions

3.12.1 Random Man Not Excluded (RMNE) – The frequency of selecting a random man from the population that could not be excluded as the biological father**.**

3.12.2 Probability of Exclusion/Power of Exclusion (PE) – The probability of excluding a random man from the population as being the biological father; is dependent on the genotypes of the child and mother and the race of the alleged father.

3.12.3 Parentage Index (PI)/Relatedness Index (RI) – A likelihood ratio based on two different conditional probabilities; PI/RI is calculated for each locus in a system.

3.12.4 Combined Parentage Index (CPI)/Combined Relatedness Index (CRI) – CPI/CRI is the product of all PIs/RIs for the loci in a system; a measure of the strength of the genetic evidence.

3.12.5 Probability of Parentage (W) – Also known as the probability of relatedness. It is the measure of the strength of the genetic evidence and the non-genetic evidence that an individual is a biological relative as compared to an unrelated, random individual in the same population.

3.12.6 Prior Probability (Pr) – The strength of the non-genetic evidence that the alleged individual is a biological relative.

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4 Y-STR Test Methods

4.1 ScopeY-STR analysis may be performed in situations where male DNA needs to be differentiated from female DNA. DNA analysis performed prior to Y-STR analysis shall be in accordance to the test methods outlined in the DNA Methods section of this document.**Precautions/Limitations**

4.2.1 Samples that may be suitable for Y-STR analysis should be evaluated by a member of the Y-STR team to determine the most appropriate sample(s) to analyze in a case. The following should be considered:

4.2.1.1 Standards from any male individuals involved in a case shall be submitted before Y-STR analysis is performed.

4.2.1.2 Cases that have not provided meaningful autosomal results should be considered for analysis.

4.2.1.3 Cases from sexual assault/misconduct should be considered for analysis.

4.2.1.3.1 Due to the limitations of the technology, property crimes generally will not be considered for analysis.

4.2.1.4 Samples with female to male ratios of greater than 10:1 should be considered for analysis.

4.3 Related Information

4.3.1 Worksheet Manual

4.4 Instruments

4.4.1 See DNA Test Methods Section 2.4

4.5 Reagents/Materials

4.5.1 See DNA Test Methods Section 2.5

4.6 Hazards/Safety

4.6.1 See DNA Test Methods Section 2.6

4.7 Reference Materials/Controls/Calibration Checks

4.7.1 See DNA Test Methods Section 2.7

4.8 Procedures/Instructions

4.8.1 See DNA Test Methods Section 2.8 for extraction procedures

4.8.1.1 Performing Y-STR analysis before or after autosomal STR analysis will be evaluated on a case by case basis.

4.8.1.2 Y-STR analysis may be performed on extracts previously used for autosomal STR testing or may require re-extraction of a sample.

4.8.1.3 Samples shall be re-quantified using Quantifiler Duo® or Plexor ® HY to get the most accurate quantification value. The male quantification value shall be used for amplification of the sample. If the male quantity is zero (undetermined) using Quantifiler Duo® the sample may be amplified.

4.8.2 Real-Time PCR Quantification for Y-STR Analysis Using Plexor® HY

4.8.2.1 Preparation Of The Quantification Plate

4.8.2.1.1 The Plexor® HY kit should be stored at -15 to -25 °C. Once thawed, the Plexor® HY Male Genomic DNA Standard shall be stored at 2 to 8 °C. Minimize the number of freeze-thaw cycles for the remaining kit components.

4.8.2.1.2 The preparation of the standard curve and the plate shall be performed in the PCR amplification set-up area.

4.8.2.1.3 A new standard curve shall be prepared for each plate.

4.8.2.1.4 **Prepare the Standard Curve**

4.8.2.1.4.1 Thaw the Plexor® HY Male Genomic DNA Standard [50 ng/µl stock]. After initial thawing, store at 2 to 8 °C.

4.8.2.1.4.2 Label seven microcentrifuge tubes Std. 1 through Std. 7.

4.8.2.1.4.3 Dispense the required amount of TE^{-4} buffer to each tube. (See table below)

4.8.2.1.4.4 Vortex the Plexor® HY Male Genomic DNA Standard for 5 seconds and add 15 µl to Std. 1.

4.8.2.1.4.5 Using a new pipette tip, add the specified amount of Std. 1 to Std. 2 and mix thoroughly.

4.8.2.1.4.6 Using a new pipette tip each time, continue diluting each successive concentration until the dilution series is complete.

Standard Curve	Dilution Series	Dilution Factor
Std. 1 [50.0 ng/µl]	0 µl TE^{-4} buffer + 15 µl of 50 ng/µl DNA stock	n/a
Std. 2 [10.0 ng/µl]	40 µl TE^{-4} buffer + 10 µl Std. 1	5X
Std. 3 [2.0 ng/µl]	40 µl TE^{-4} buffer + 10 µl Std. 2	5X
Std. 4 [0.4 ng/µl]	40 µl TE^{-4} buffer + 10 µl Std. 3	5X
Std. 5 [0.08 ng/µl]	40 µl TE^{-4} buffer + 10 µl Std. 4	5X
Std. 6 [0.016 ng/µl]	40 µl TE^{-4} buffer + 10 µl Std. 5	5X
Std. 7 [0.0032 ng/µl]	40 µl TE^{-4} buffer + 10 µl Std. 6	5X

4.8.2.1.5 **Prepare the Reactions**

4.8.2.1.5.1 Each plate shall contain a standard curve series run in duplicate and at least one Non-Template Control (NTC).

4.8.2.1.5.2 Determine the number of samples to be quantified, including standards and the non-template control(s). Add 3 to 5 reactions to this number to compensate for the loss that occurs during reagent transfers.

4.8.2.1.5.3 Using the Plexor® HY worksheet, calculate the required amount of each component of the master mix. Multiply the volume per reaction (µl) by the total number of reactions.

PCR Master Mix Component	Volume per Reaction (µl) (assumes 2 µl template per reaction)
Plexor® HY 2X Master Mix	10.0 µl
Water, Amplification Grade	7.0 µl
Plexor® HY 20X Primer/IPC Mix	1.0 µl
Total Volume	18.0 µl

4.8.2.1.5.4 Thaw the Plexor® HY 2X Master Mix, Plexor® HY 20X Primer/IPC Mix, and Water, Amplification Grade.

4.8.2.1.5.5 Vortex the 2X Master Mix and 20X Primer/IPC Mix for 3 to 5 seconds. Do not centrifuge as this may cause primers to be concentrated at the bottom of the tube.

4.8.2.1.5.6 Combine the required volumes of Water, Amplification Grade, 2X Master Mix, and 20X Primer/IPC Mix into a microcentrifuge tube.

4.8.2.1.5.7 Vortex briefly to mix and centrifuge.

4.8.2.1.5.8 Dispense 18 µl of the Master Mix into each reaction well of a 96-well optical plate.

4.8.2.1.5.9 Add 2 µl of sample, standard, or Non-Template Control (TE^{-4}) to the appropriate wells.

4.8.2.1.5.10 Seal the reaction plate with an Optical Adhesive Cover. Run the edge of the cover applicator between the rows and columns of the wells to ensure that all wells are sealed properly.

4.8.2.1.5.11 Spin the plate using either a centrifuge or a salad spinner to remove any bubbles and force samples into the bottom of each well.

4.8.2.2 Create Plate Document and Run Plate (Note: The plate document shall be created and saved on the computer prior to running the quantification plate on the instrument.)

4.8.2.2.1 Turn on the computer.

4.8.2.2.2 Turn on the Real-Time PCR instrument (must be on prior to opening the software).

4.8.2.2.3 Open the Applied Biosystems 7500 System Sequence Detection Software v 1.2.3.

4.8.2.2.4 Set up the Plate Document as described below: File ⇒New

Assay: Absolute Quantification (Standard Curve)
Container: 96-Well Clear
Template: Plexor Template
Operator: your name
Plate Name: ”First lab file on plate_date_plate#” ex. “00A1234_01Jan11_01”

4.8.2.2.5 Click Finish.

4.8.2.2.6 Ensure that detectors (Autosomal, IPC and Y) are selected only for wells that are being used. Including unused wells will significantly impact the scale of the X and Y axes when viewing the data.

4.8.2.2.7 Sample names should be added later within the Plexor® Analysis Software.

4.8.2.2.8 Save the plate document at this time.

4.8.2.2.9 Place the plate in the instrument.

4.8.2.2.10 Select the **Instrument** tab of the plate document and click “Start”.

4.8.2.2.11 When the run is finished, click “Okay”.

4.8.2.3 Analyze the Plate

4.8.2.3.1 In the SDS software, analyze the plate by clicking the Green Arrow (►) on the toolbar or select Analysis ⇒ Analyze.

4.8.2.3.2 To export the amplification data, select File ⇒ Export ⇒ Delta RN. Save the .csv file with an appropriate name (First lab file on plate_date_plate#_DeltaRn).

4.8.2.3.3 To export the melt/dissociation data, select File ⇒ Export ⇒ Dissociation ⇒ Raw and Derivative Data. Save the .csv file with an appropriate name (First lab file on plate_date_plate#_melt). **Note:** When a dissociation curve is included in a thermal cycling program, the SDS software may expect SYBR® green as the dye choice. A message will appear when analyzing an experiment. Select “Yes” and continue.

4.8.2.4 Data Import into the Plexor® Analysis Software

4.8.2.4.1 To launch the Plexor® Analysis Software (forensic release) go to the Start menu ⇒ All Programs ⇒ Plexor. A shortcut can be placed on the desktop by going to the Start menu ⇒ Programs, then right-click on Plexor ⇒ Create Shortcut.

4.8.2.4.2 In the File menu, deselect “Set Passive Reference On Import”.

4.8.2.4.3 the File menu, select “Import New Run”, or select the icon: 

4.8.2.4.4 Select “Applied Biosystems 7500 SDS v1.4 & prior” as the instrument type.

4.8.2.4.5 Select “Add Target” three times. For each dye, assign a target name, enter the correct dye name (FL for Autosomal, CO560 for Y and CR610 for IPC) and indicate that there are amplification data and melt (dissociation) data to be analyzed for each dye.

4.8.2.4.6 Select “Next”.

4.8.2.4.7 Enter Operator Name.

4.8.2.4.8 Select “Next”.

4.8.2.4.9 Use “Browse” on the File Import Screen to specify the amplification and dissociation/melt data files previously exported from the instrument.

4.8.2.4.10 Select “Finish” to complete the data import and open the Analysis Desktop.

4.8.2.5 Data Analysis with Plexor® Analysis Software

4.8.2.5.1 Sample Definition.

4.8.2.5.1.1 Define the DNA standards.

4.8.2.5.1.1.1 Select the **PCR Curves** tab and use the well selector to highlight the wells that contain the DNA standards.

4.8.2.5.1.1.2 Select the Dilution Series icon:  .

4.8.2.5.1.1.3 Confirm that the series selected is the “Vertical Series” and the series is “Decreasing”. Enter 50 for the starting concentration and 5 for the dilution factor.

4.8.2.5.1.1.4 Select “Apply”.

4.8.2.5.1.2 Define the Non-Template Control reactions.

4.8.2.5.1.2.1 Select the **PCR Curves** tab and use the well selector to highlight the wells that contain NTC reactions.

4.8.2.5.1.2.2 Select the NTC icon:  .

4.8.2.5.1.3 Assign Sample Names

4.8.2.5.1.3.1 Select the **Sample IDs** tab.

4.8.2.5.1.3.2 Select the well and enter the desired sample name for each sample. Each sample shall be uniquely indentified.

4.8.2.5.1.3.3 Select “Accept Changes”.

4.8.2.5.2 Adjust Target Melt Temperature.

4.8.2.5.2.1 Select the **PCR Curves** tab.

4.8.2.5.2.2 Change the autosomal melt temperature range by clicking on the blue shaded area on the graph (default temperature is set at 90.0).

4.8.2.5.2.3 In the **melt curves** window, move the mouse so that the arrow is over the expected melt temperature line and drag the range to approximately the middle of the melt curves.

4.8.2.5.2.4 Repeat for Y and IPC by choosing the appropriate **PCR Curves** tab.

4.8.2.5.3 Standard Curves and Sample Concentrations

4.8.2.5.3.1 To generate the autosomal standard curve, select the **Autosomal** tab in the **PCR Curves** tab and select the standards in the well selector.

4.8.2.5.3.2 Select the Add Standard Curve icon:  .

4.8.2.5.3.3 Select the **Y** tab in the **PCR Curves** tab and repeat to generate the Y standard curve.

4.8.2.5.3.4 To view the standard curves, select the **Standard Curves** tab.

4.8.2.5.3.5 To print the autosomal and Y standard curves, select “Print a Screenshot” from the File menu.

4.8.2.5.3.6 Examine the autosomal and Y standard curves. (See Interpretation Guidelines for Plexor® HY Real-time PCR Quantification for further evaluation of the standard curves.)

4.8.2.5.3.6.1 Autosomal slope acceptable range:-3.1 to-3.7

4.8.2.5.3.6.2 Y slope acceptable range: -3.0 to -3.6

4.8.2.5.3.6.3 R^2 value shall be ≥0.98

4.8.2.5.3.6.4 Autosomal Y-intercept acceptable range: 22 – 25.

4.8.2.5.3.6.5 Y Y-intercept acceptable range: 23 – 26.

4.8.2.5.3.7 To obtain autosomal quantification values for all samples, select the **Autosomal** tab in the **PCR Curves** tab and select all samples and standards in the well selector.

4.8.2.5.3.8 Select the Add Standard Curve icon:  .

4.8.2.5.3.9 Select the **Y** tab in the **PCR Curves** tab and repeat to obtain the Y quanitification values for all samples.

4.8.2.5.4 Generate Forensic Report (without normalization).

4.8.2.5.4.1 In the Forensic Menu, select “Normalization and IPC Parameters”.

4.8.2.5.4.2 Check the “disable volume normalization (show concentrations and Cq values only)” box.

4.8.2.5.4.3 Select the **Internal PCR Control (IPC)** tab and change the IPC threshold value to 0.7.

4.8.2.5.4.4 Select “OK”.

4.8.2.5.4.5 Select the **Reports** tab and then the **Forensics** tab to view results.

4.8.2.5.4.6 Select all samples in the Forensic Report table and right click on the outside of the table.

4.8.2.5.4.7 Select “Change Columns Shown” and check the “IPC Observed Cq” and the “IPC Expected Cq” boxes.

4.8.2.5.4.8 Check for inhibition of samples by evaluating the IPC Status and IPC values on the Forensic Report.

4.8.2.5.4.8.1 The IPC status indicates if the difference between the sample’s C_T for the IPC and the DNA standard is greater than the 0.7. A difference of less than 0.7 is indicated as “OK” while a difference greater than 0.7 is indicated as “Check IPC”.

4.8.2.5.4.9 Check the “Curves Status” for the samples on the Forensic Report.

4.8.2.5.4.9.1 “OK” indicates the following:

4.8.2.5.4.9.1.1 The sample, if defined as a standard, shows amplification.

4.8.2.5.4.9.1.2 The sample, if defined as a non-template control, shows no amplification.

4.8.2.5.4.9.1.3 If a melt peak is present, the T_m is within the expected range.

4.8.2.5.4.9.2 “Check STD”, “Check NTC”, or “Check Melts” will be displayed if the above criteria or not met.

4.8.2.5.4.10 Print Forensic Report.

4.8.2.5.4.10.1.1 Select all samples in the Forensic Report.

4.8.2.5.4.10.1.2 Select the Export Selected icon:  .

4.8.2.5.4.10.1.3 Save the .tab file with an appropriate name (First lab file on plate_date_plate#_ForensicReport).

4.8.2.5.4.10.1.4 Open the .tab file using Microsoft Excel and print Forensic Report.

4.8.2.5.5 Save the analysis file (.aan) with an appropriate name (First lab file on plate_date_plate#) by selecting "Save Analysis File (.aan)" in the File menu.

4.8.2.5.6 Save required data under each associated laboratory case number and request folder located in the analysts' folders on the server. The required data include the Applied Biosystems 7500 System Sequence Detection Software .sds file, the exported amplification and melt/dissociation data (.csv), the Plexor® Analysis Software Forensic Report (.tab), and the Plexor® Analysis Software Analysis File (.aan).

4.8.2.5.7 At a minimum the required documents for the case record shall be generated. The required documents include the exported Microsoft Excel Forensic Report, the autosomal standard curve and the Y standard curve.

4.8.2.6 Interpretation Guidelines for Plexor® HY Real-Time PCR Quantification

4.8.2.6.1 **Controls**

4.8.2.6.1.1 **Standard Curve**: The purpose of the standard curve is to evaluate the quality of the results from the quantification standard reactions.

4.8.2.6.1.1.1 **Slope:** Indicates the PCR amplification efficiency for the assay. A slope of -3.3 indicates 100% amplification efficiency. Scientific rounding rules apply. The analyst shall obtain documented Technical Leader approval to use data when the slope is outside the accepted range.

	Range
Plexor® HY Autosomal	-3.1 to -3.7
Plexor® HY Y	-3.0 to -3.6

4.8.2.6.1.1.2 **R^2 value:** a measure of the closeness of fit between the standard curve regression line and the individual C_T data points of the quantification standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points. This value shall be ≥ 0.98. If the R^2 value is <0.98 the test is inconclusive and should be repeated. The Technical Leader shall be notified with documentation in the case record.

4.8.2.6.1.1.3 **Y Intercept:** indicates the expected C_T value for a sample with Quantity = 1 (i.e. 1 ng/µl). The analyst shall obtain documented Technical Leader approval to use

data when the Y-intercept value is outside of the acceptable range.

	Range
Plexor® HY Autosomal	22 to 25
Plexor® HY Y	23 to 26

4.8.2.6.1.1.4 Occasionally there will be an outlier data point on the standard curve. Low R^2 values (<0.98) may be due to variability for replicate samples of the 0.0032 ng/µl dilution. The 0.0032 ng/µl standard may show increased variability compared to the other dilutions, most notably in autosomal reactions. The 0.0032 ng/µl standard(s) may be removed from the autosomal and/or Y standard curves by changing the standard(s) to "unknown".

4.8.2.6.1.1.5 One other data point in addition to the 0.0032 ng/µl standard(s) may be removed from each standard curve. The analyst shall have documented supervisor approval to omit additional points in the standard curve(s). The Technical Leader shall be notified with documentation in the case record.

4.8.2.6.1.2 **Non-Template Control (NTC):** Contains PCR reagent, but no template DNA. Occasionally, a value may be given for the NTC due to background fluorescence. The analyst may proceed with amplification and typing of the samples using caution with interpretation of any profiles obtained from the samples.

4.8.2.6.1.3 **Internal PCR Control (IPC):** The purpose of the IPC is to distinguish between a true negative sample result and reactions affected by the presence of PCR inhibitors, assay setup, and/or chemistry/instrument failure.

4.8.2.6.1.3.1 An inhibition check can be done by comparing IPC C_T values for unknown samples with IPC C_T values for DNA standards. The set IPC threshold is 0.7 cycles. If a sample's IPC C_T value is more than 0.7 cycles greater than a comparable standard, there may be an indication of inhibition by the sample. High levels of total human DNA (>10 ng/µl) may cause a slight delay (1 – 2 cycles) the IPC crossing the cycle threshold. Given this information, an analyst may choose to dilute, Microcon® and/or centri-sep the sample prior to amplification and typing.

4.8.2.6.1.3.2 An undetected IPC C_T may also be an indication of inhibition. The analyst may choose to dilute, Microcon® and/or centri-sep the sample before continuing with analysis. It is at the discretion of the analyst whether or not to continue processing a sample in which the IPC is undetected. If the sample is not carried throughout the testing process, it shall be reported out as inconclusive.

4.8.2.6.2 **Amplification/Melt Curves**

4.8.2.6.2.1 If "Check STD" is displayed for the Curves Status, ensure the standard curve has been properly generated. See Sections 4.8.2.6.1.1.4 and 4.8.2.6.1.1.5 for deleting points from the standard curve(s).

4.8.2.6.2.2 If "Check NTC" is displayed in the Curves Status and the NTC has a value, the samples may be amplified (see Section 4.8.2.6.1.2).

4.8.2.6.2.3 If "Check Melts" is displayed in the Curves Status or the melt curve for a sample is not within the expected range, the sample shall still be amplified. A shift in the melt curve may indicate non-specific amplification, primer dimers, or contaminant DNA.

Target	T_m Range
Autosomal	79-81 °C
Y	81-83 °C
IPC	79-81 C

The IPC T_m value can fall as much as 2 °C outside the normal range. The default target range is T_m +/- 1.5 °C.

4.8.2.6.2.4 A "No Call" for the T_m indicates that the melt curve displays the expected melt temperature, but there is insufficient amplification product to cause the melt curve to cross the melt threshold. If the amount of male DNA present exceeds the set threshold, additional Y-STR analysis of the sample shall be performed.

4.8.2.6.2.5 It is acceptable for the 0.0032 ng/µl DNA standard to display "No" or "No Call" in the "Tm?" column. Verify that if any subthreshold peak is present in the melt curve, this peak is within the expected target melt temperature range.

4.8.2.6.3 **Dynamic Range**

4.8.2.6.3.1 If the quantity of male DNA detected in the sample is below 0.001 ng/µl, no additional Y-STR analysis of that sample shall be performed.

4.8.2.6.3.2 If the quantity of male DNA in a sample is > 50 ng/µl, dilutions shall be made and re-quantified if they are to be amplified.

4.8.2.6.3.3 Quantification values from the Y target shall be used for amplification of Y-STR's.

4.8.2.6.3.4 Because the quantification values may have many significant figures, the analyst may truncate the value to one digit past the decimal point, but only after the value has been multiplied by 5 for amplification. (i.e. 1A = 1.59 ng/µl x 5 µl = 7.95 ng/5 µl = 7.9).

4.8.3 PowerPlex® Y Introduction (Applied Biosystems 3130xl Genetic Analyzer)

4.8.3.1 Short tandem repeat (STR) markers on the Y chromosome (Y-STR) have qualities that are distinct from autosomal markers and are useful for human identification. The Promega PowerPlex® Y System allows co-amplification and three-color detection of twelve loci. The amplification occurs in a single reaction tube and detection occurs by a single capillary electrophoresis injection.

4.8.3.2 The Applied Biosystems 3130xl Genetic Analyzer utilizes electrokinetic injection of DNA molecules into polymer-filled capillaries which separates the DNA fragments by size. The fluorescent tag labeled primers incorporated into the PowerPlex® Y amplification products are responsive to the frequency of the 15 mW argon-ion laser. Upon excitation, the fluorophores are raised to a higher energy level. When the fluorophores return to their normal energy level, a fluorescent signal is emitted. This signal is then detected by a camera within the 3130xl capillary electrophoresis instrument which converts the signal to a computer image where it is visualized in an electropherogram as a peak.

4.8.3.3 The data produced by the 3130xl Genetic Analyzer is analyzed with GeneMapper ® *ID-X* Software which results in peaks labeled with their allele designation. The allele designation for each sample is accomplished through the use of an internal lane standard (ILS). The ILS is injected with each sample and it contains 22 fragments of known length. The ILS determines the base pair size of the fragments in the sample and the software compares the sizes to an allelic ladder to determine the allele designation.

The PowerPlex ® Y System Locus-Specific and Allelic Ladder Information

Locus	Chromosomal Location	Repeat Sequence[1] 5'-->3'	Size Range of Allelic Ladder Components (bases)	STR Ladder Alleles (# of repeats)	Fluorophore
DYS391	Yq	TCTA (14)	90-118	6, 8-13	Fluorescein
DYS389 I/II	Yq	[TCTG][TCTA] Complex (14)	148-168 (I) 256-296 (II)	10-15 (I) 24-34 (II)	Fluorescein
DYS439	Yq	GATA (26)	203-231	8-15	Fluorescein
DYS393	Yq	AGAT (14)	104-136	8-16	TMR
DYS390	Yq	[TCTG][TCTA] Complex (14)	191-227	18-27	TMR
DYS385 a/b	Yq	GAAA (14)	243-315	7-25	TMR
DYS438	Yq	TTTTC (26)	101-121	8-12	JOE
DYS437	Yq	[TCTA][TCTG] Complex (26)	183-199	13-17	JOE
DYS19	Yq	TAGA Complex (14)	232-268	10-19	JOE
DYS392	Yq	TAT (14)	294-327	7-18	JOE

TMR = carboxy-tetramethylrhodamine
JOE = 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein

[1] The August 1997 report (27, 28) of the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) states, "1) for STR loci within coding genes, the coding strand shall be used and the repeat sequence motif defined using the first possible 5´ nucleotide of a repeat motif; and 2) for STR loci not associated with a coding gene, the first database entry or original literature description shall be used".

4.8.4 **PowerPlex® Y Amplification Set-Up** (Note: The following steps shall be performed in the PCR amplification set-up area.)

4.8.4.1 Thaw the AmpliTaq Gold® Polymerase, Gold ST★R 10X Buffer, and PowerPlex® Y 10X Primer Pair Mix. When thawed, it is important to vortex the Gold ST★R 10X Buffer and PowerPlex® Y 10X Primer Pair Mix tubes for 5-10 seconds. (Do not centrifuge the 10X Primer Pair Mix as this may cause the primers to be concentrated at the bottom of the tube.)

4.8.4.2 Determine the number of samples to be amplified, including controls (reagent blank, positive control [PC], female negative control [NC] and amplification blank [AB]). Add 2 to 4 reactions to this number to compensate for the loss that occurs during reagent transfers.

4.8.4.3 Place one 0.2 ml reaction tube for the Model 9700 Thermal Cycler for each sample into a rack, label appropriately.

4.8.4.4 Using the Master Mix worksheet, calculate the required amount of each component of the PCR master mix. Multiply the volume (μl) per

sample by the total number of reactions (from 4.8.3.3) to obtain the final volume (µl).

4.8.4.4.1 Components of Master Mix/sample:

4.8.4.4.2 14.4µl Nuclease Free Water

4.8.4.4.3 2.5µl Gold ST★R 10X Buffer

4.8.4.4.4 2.5µl 10X PowerPlex® Y Primer Pair Mix

4.8.4.4.5 0.6µl AmpliTaq Gold® (4U @ 5U/µl)

4.8.4.4.6 20µl Total Volume (w/o sample)

4.8.4.5 Add the calculated volume of each component to a 1.5 ml tube. Mix gently.

4.8.4.6 Add 20 µl of PCR master mix to each sample tube using a positive displacement pipettor or a repeat pipettor.

4.8.4.7 Pipette 5.0 µl of each sample into the respective tube containing Master Mix. 0.4 to 1.0 ng of template DNA is recommended. (Amplification of greater than 1.0 ng of template DNA should not be used due to off-scale peak heights and imbalance in peak heights from locus to locus.) Amplification of less than 0.4 ng can be used with caution. **Note:** For organic extractions if the template DNA is stored in TE buffer, the volume of the DNA sample added should not exceed 20% of the final reaction volume. PCR amplification efficiency and quality can be greatly altered by changes in pH (due to added Tris-HCl) or available magnesium concentration (due to chelation by EDTA). DNA samples stored (or diluted) in NFH_2O are not subject to this caution, but may contain other PCR inhibitors at low concentrations depending on the source of the template DNA and the extraction procedure employed. If nuclease-free water was used in the final wash and collection step of the extraction, the volume of sample added to the amplification reaction can vary from 5.0 µl to 19.4 µl. The difference in the final volume shall then be subtracted from the nuclease-free water added to the PowerPlex® Y amplification master mix.

4.8.4.8 For the positive control, dilute the male DNA standard supplied with the PowerPlex® Y kit to 0.08 to 0.2ng/µl. Pipette 5.0µl (0.4 to 1.0ng) of diluted male DNA into a 0.2 µl reaction tube containing 20 µl of PCR master mix. A positive control shall be included in each thermal cycler.

4.8.4.9 For the female negative control, dilute the female DNA standard supplied with the PowerPlex® Y kit or other appropriate female standard to 0.08 to 0.2ng/µl. Pipette 5.0µl (0.4 to 1.0ng) of diluted female DNA into a 0.2 µl reaction tube containing 20µl of PCR master mix. A negative control shall be included in each thermal cycler.

4.8.4.10 For the negative amplification control, pipette 5.0µl of nuclease free water into a 0.2 µl reaction tube containing 20µl of the PCR master mix. A negative amplification control shall be included in each thermal cycler.

4.8.5 PowerPlex® Y Amplification

4.8.5.1 Assemble the tubes in a thermal cycler.

4.8.5.2 Select and run the thermal cycling protocol below:

4.8.5.3 Applied Biosystems GeneAmp® 9700 Thermal Cycler

4.8.5.3.1 95°C for 11 minutes, then:

4.8.5.3.2 96°C for 1 minute, then:

4.8.5.3.3 Ramp 100% to 94°C for 30 seconds

4.8.5.3.4 Ramp 29% to 60°C for 30 seconds

4.8.5.3.5 Ramp 23% to 70°C for 45 seconds

4.8.5.3.6 for **10 cycles**, then:

4.8.5.3.7 Ramp 100% to 90°C for 30 seconds

4.8.5.3.8 Ramp 29% to 58°C for 30 seconds

4.8.5.3.9 Ramp 23% to 70°C for 45 seconds

4.8.5.3.10 for **22 cycles**, then:

4.8.5.3.11 60°C for 30 minutes, then:

4.8.5.3.12 Soak at 4°C until the tubes are removed

4.8.5.4 **Note:** When programming the GeneAmp® PCR System 9700 thermal cycler, use the ramping mode for the GeneAmp® PCR System 9600 thermal cycler.

4.8.5.5 Remove samples after the amplification process is completed.

4.8.5.6 Store the amplified samples in the freezer or refrigerator (if they are to be used within 2 days).

4.8.6 Applied Biosystems 3130xl Genetic Analyzer - Data Collection Software version 3.0 - Instrument Set-up

4.8.6.1 Creating a Run Module, Protocol, and Results Group (**only necessary the first time PowerPlex® Y samples are run or if the parameters change**.)

4.8.6.2 In the “Module Manager,” select “New.” Select “Regular” in the “Type” drop-down list, and select “HIDFragmentAnalysis36_POP4” in the “Template” drop-down list. Confirm that the injection time is 5 seconds and the injection voltage is 3 kV. Lengthen the run time to

2,000 seconds. Name the run module with the kit name, injection voltage and injection time and select "OK". Additional validated injection protocols may be utilized. The acceptable injection protocols are listed within the Indiana State Police Laboratory Forensic Biology Casework Test Method under *General Rules for PowerPlex Y® Analysis on the Applied Biosystems 3130XL Genetic Analyzer*. (Injection protocols Ys) A run module and instrument protocol shall be created for each injection time protocol needed.

4.8.6.3 In the "Protocol Manager," select "New." Name the protocol with the kit name, injection voltage and injection time. Select "Regular" in the "Type" drop-down list, and select the appropriate run module from the "Run Module" drop-down list. Select "F" in the "Dye-Set" drop down list. Select "OK."

4.8.6.4 In the "Results Group Manager", select "New." Select the "General" tab and enter "PowerPlex Y" for the name. Select the "Analysis" tab, and select "GeneMapper—Generic" in the "Analysis Type" drop-down list. Leave the destination as "E:\Applied Biosystems\udc\datacollection\data." Select the "Naming" tab and change the Prefix to "PPY". Under Format, select "Well Position" and "Sample Name" in the drop-down lists. Under the options for the Run Folder Name, select "Plate Name" from the drop-down lists. Under the Automated Processing tab select that Autoanalysis is performed "Only when the results group is complete."

4.8.7 Applied Biosystems 3130xl Genetic Analyzer - Data Collection Software version 3.0 - PowerPlex® Y Electrophoresis

4.8.7.1 Sample Preparation

4.8.7.1.1 **Note:** The quality of formamide is critical for the successful detection of a DNA profile. Deionized formamide shall be used that has a conductivity of less than 100µS/cm, such as Hi-Di™ Formamide. The formamide shall be frozen in aliquots at -20°C and the remainder of each aliquot shall be discarded after it is thawed. Multiple freeze-thaw cycles or long-term storage at 4°C may cause breakdown of the formamide which can create ions that compete with DNA during injection. This will cause lower peak heights and decreased sensitivity.

4.8.7.1.2 **Caution:** Formamide is an irritant and teratogen; therefore universal precautions and a fume hood shall be utilized when working with formamide to avoid inhalation and contact with the skin.

4.8.7.1.3 Thaw the ILS 600, the allelic ladder, and an aliquot of Hi-Di™ formamide. When thawed, vortex to mix.

4.8.7.1.4 Determine the number of samples to be injected, including controls (reagent blanks, positive control, female negative control and amplification blank) and allelic ladders. Add 2 to 4 reactions

to this number to compensate for loss that occurs during reagent transfers.

4.8.7.1.5 Prepare a loading cocktail by combining the internal lane standard (ILS 600) with the Hi-Di™ formamide as follows:

4.8.7.1.6 [(0.5 µl ILS 600) x (# samples)] + [9.5 µl Hi-Di™ formamide) x (# samples)]

4.8.7.1.7 The volume of ILS 600 used in the loading cocktail may be decreased to optimize size standard peaks. The optimal range of peak heights for the size standard should be around 400 to1000 relative fluorescent units (RFU). If the peak heights of the size standard are too high, pull-up of the size standard peaks into the other dyes can occur. Optimization of size standard peaks to limit the observation of pull-up while maintaining sufficient peak heights can be obtained by using 0.5 to 1.0 µl of ILS 600 per well. The amount of Hi-Di™ formamide shall be adjusted so that the total amount of loading cocktail for each well is 10 µl.

4.8.7.1.8 Vortex to mix.

4.8.7.1.9 Pipette 10 µl of the formamide/ILS 600 mixture into each well. (Add formamide or formamide/ILS mixture into empty wells to complete an injection set of sixteen. Every well in which an injection is occurring must contain liquid.)

4.8.7.1.10 Add 1 µl of amplified sample or 1 µl of the allelic ladder mix to each well. It is recommended that one allelic ladder is injected within every 32 samples on a sixteen capillary instrument and one allelic ladder to every 16 samples on a four capillary instrument to ensure that a usable ladder injection occurs. At least one allelic ladder is required within each run folder.

4.8.7.1.11 Cover the wells with the plate septa and briefly spin down to remove air bubbles from the wells.

4.8.7.1.12 Denature the samples at 95^{o}C for ~3 minutes, then immediately chill on crushed ice or a cold pack for ~3 minutes. Denature the samples just prior to loading the instrument. Avoid denaturing the samples for longer than 3 minutes as extended heat denaturing can lead to the appearance of artifacts.

4.8.7.2 Creating a Plate Record

4.8.7.2.1 Y-STR analysis shall be performed on an instrument that has been performance checked and labeled for use with Y-STRs.

4.8.7.2.2 In the “Plate Manager,” select “New.” Name the plate record “Laboratory case number_date_ the injection number of the plate” (ex. 00A1234_01Jan11_01). If more than one laboratory case number is loaded onto a plate, use the first file number injected in the plate name. The first injection of a prepared plate shall be 01; if the plate is re-injected the number shall increase sequentially. If

the same plate preparation is run on a different date, the original date should still be used from when the plate was prepared. If a second preparation of a plate is made on the same date as the first, it shall be designated with a "-2" after the date. (ex. 00A1234_01Jan11-2_01). In the "Application" drop-down list select "GeneMapper—Generic", and select 96-well in the plate type drop-down list. Add your name or initials in the owner and operator windows, and select "OK".

4.8.7.2.3 In the plate record, enter sample names in the appropriate cells and scroll to the right. The sample name shall include the sample sub-item as well as the laboratory case number (ex. 1A1_00A1234 if more than one case is included on the plate). If only one case is present on the plate then only the subitem number is required. In order to aid in GeneMapper® *ID-X* sample analysis, it is recommended to place a "z" in front of the sample name for known standards (ex. z1A1_00A1234") here or in GeneMapper® *ID-X* under sample name. In the "Results Group 1" column, select the "PowerPlex Y" results group from the drop-down list. In the "Instrument Protocol 1" column select the appropriate protocol from the drop-down list. Fill in these selections for each row that contains a sample name. Note: the instrument will inject in groups of 16 regardless of whether there is a sample name. Be sure all wells in a group have Hi-Di™ present.

4.8.7.2.4 If more than one run is needed for any given sample, select "Edit", then "Add Sample Run" and fill in the appropriate Results Group and Instrument Protocol entries. If only specific samples are to be run with a second protocol, only those samples to be analyzed need to contain a result group and protocol.

4.8.7.2.5 Fill out a Plate Record Worksheet for each plate. The original plate record worksheet shall remain in a log book at the instrument work station. A copy of the worksheet shall be maintained in the case record.

4.8.7.3 Starting the Plate Run

4.8.7.3.1 Check that daily maintenance activities have been performed (i.e. buffer and dH_2O reservoirs have been rinsed and re-filled) and that no bubbles are present in the polymer delivery area. If bubbles are present, run the "Bubble Remove Wizard."

4.8.7.3.2 Place the prepared and assembled plate on the autosampler.

4.8.7.3.3 In the "Run Scheduler", click "Find All" to bring up the list of plate records. To link the plate to the plate record, select the plate record from the list, then click the plate position indicator. The plate position indicator will change from yellow to green. Click the

green “Run Instrument” arrow, and then click "OK" in the start processing dialog.

4.8.7.3.4 Record the run information in the 3130 Run Log.

4.8.7.3.5 When the run is complete, remove the plate from the instrument and store in the freezer until you have checked all of the sample results to ensure that no samples need to be re-injected.

4.8.8 See DNA Test Methods Section 2.8.18 for GeneMapper® *ID-X* Version 1.2 Software User Accounts and Security Setting procedures.

4.8.9 GeneMapper® *ID-X* Version 1.2 Software - PowerPlex® Y Software Settings

4.8.9.1 Importing Panel and Bin Files

4.8.9.1.1 Open the GeneMapper® *ID-X* Version 1.2 software.

4.8.9.1.2 Select “Tools” then “Panel Manager”.

4.8.9.1.3 Highlight the “Panel Manager” icon in the navigation pane.

4.8.9.1.4 Select “File” then “Import Panels”.

4.8.9.1.5 Navigate to the saved panel, bin, and stutter files. Select “PowerPlex_Y_ISP_Panels_IDX_v2.0” then click “Import”.

4.8.9.1.6 Select the “ISP Casework Security Group”. Click “OK”.

4.8.9.1.7 In the navigation pane, highlight the “PowerPlex_Y_ISP_Panels_IDX_v2.0” folder.

4.8.9.1.8 Select “File”, then “Import Bin Set”.

4.8.9.1.9 Select “PowerPlex_Y_ISP_Bins_IDX_v2.0” then click “Import”.

4.8.9.1.10 In the navigation pane, highlight the “PowerPlex_Y_ISP_Panels_IDX__v2.0” folder.

4.8.9.1.11 Select “File”, then “Import Marker Stutter”. A warning box will appear asking to overwrite the current values. Select “Yes”.

4.8.9.1.12 Select “PowerPlex_Y_ISP_Stutter_IDX_v2.0” then click “Import”. This will import the Promega Marker Stutter file that has been modified to include the Indiana State Police PowerPlex® Y stutter precentages for filtering out stutter as determined by the internal validation studies.

4.8.9.1.13 In the Panel Manager window, select “Apply”, then “OK”.

4.8.9.2 Creating a Casework Analysis Method

4.8.9.2.1 Select “Tools”, then “GeneMapper® *ID-X* Manager”.

4.8.9.2.2 Select the Analysis Methods tab.

4.8.9.2.3 Select “New” and a new analysis method dialog box will open.

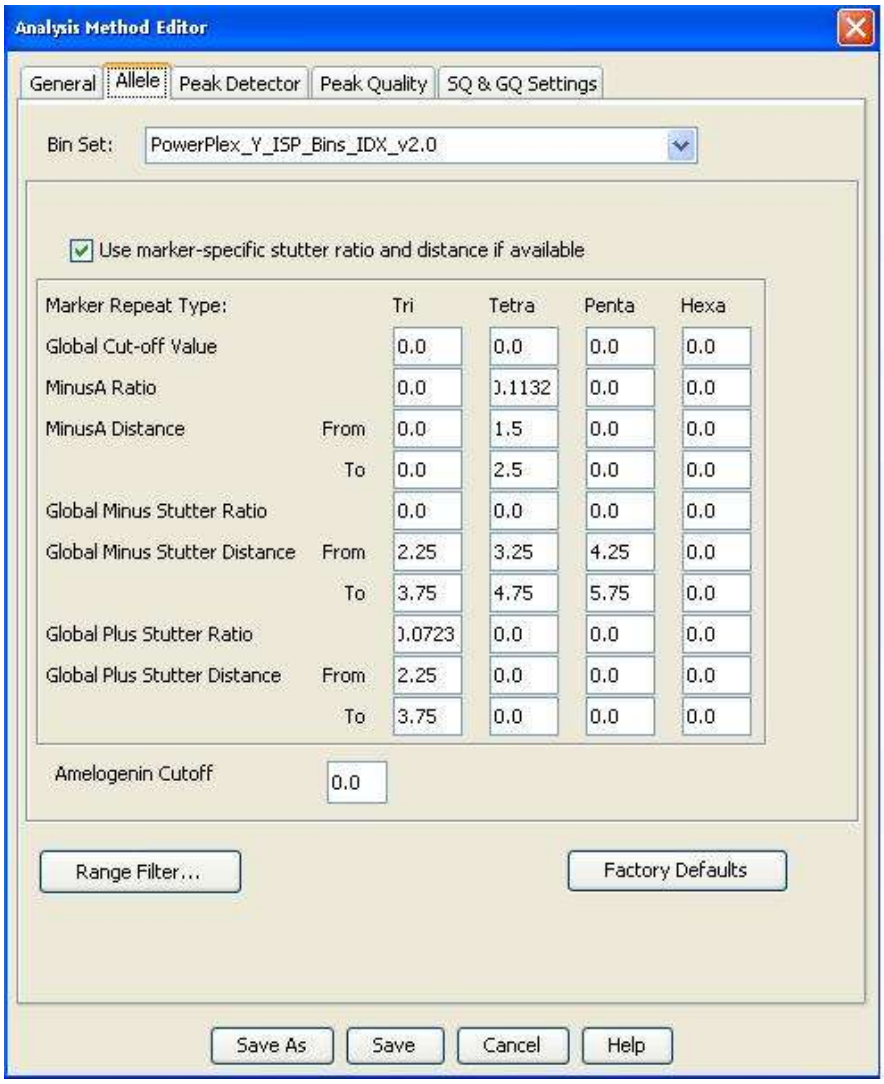
4.8.9.2.4 Enter the name “PowerPlex Y”.

4.8.9.2.5 Select the “ISP Casework Security Group”.

4.8.9.2.6 Enter 3130 as the instrument.

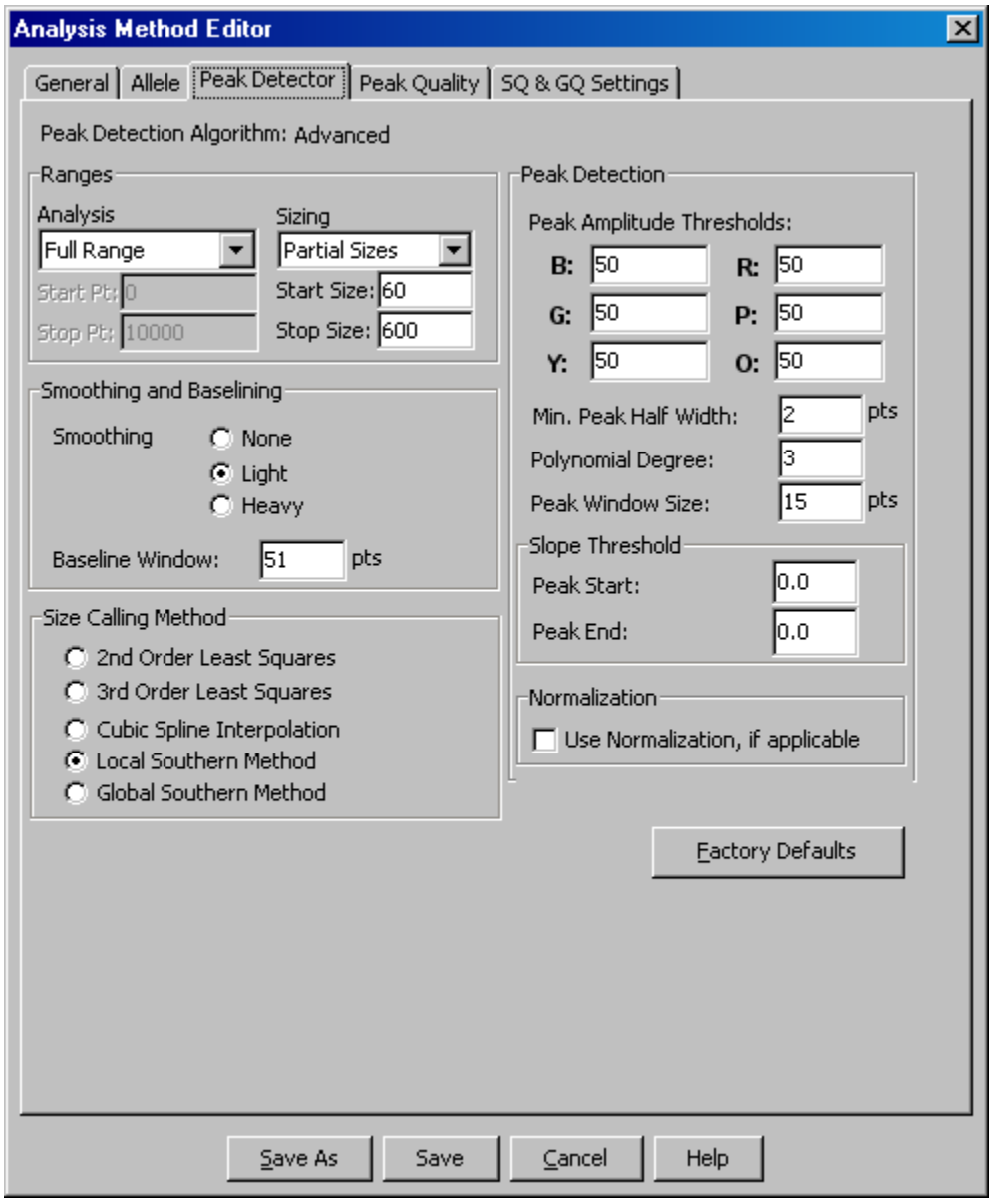
4.8.9.2.7 Select the “Allele” tab. In the “Bin Set” drop-down menu select “PowerPlex_Y_ISP_Bins_IDX_v2.0”. Ensure that the “Use marker-specific stutter ratio if available” box is checked. Enter the values shown in Figure 1 for proper filtering of stutter peaks.

Figure 1: The Allele Tab



4.8.9.2.8 Select the “Peak Detector” tab. Change the settings to match Figure 2.

Figure 2: The Peak Detector Tab.



4.8.9.2.9 The Peak Amplitude Threshold (analytical threshold) values were determined during the internal validation by calculating the noise level of each instrument and determining the cut-off in which the majority of the noise peaks would be below.

4.8.9.2.10 Select the “Peak Quality” tab. Change the settings to match Figure 3.

Figure 3: The Peak Quality Tab

4.8.9.2.11 Leave the “SQ and GQ Settings” tab set to the factory defaults.

4.8.9.2.12 Select “Save”.

4.8.9.3 Creating a Size Standard

4.8.9.3.1 The “ILS HS” size standard will be used (see 2.8.19.3 for exact settings and sizes).

4.8.9.4 Creating a Table Setting

4.8.9.4.1 The “PP16 HS” table setting will be used (see 2.8.19.4 for exact settings).

4.8.9.5 Creating a Plot Setting – Samples, Controls, and Ladders

4.8.9.5.1 The "PP16 HS" plot setting will be used (see 2.8.19.5 for exact settings).

4.8.10 GeneMapper® *ID-X* Version 1.2 Software - PowerPlex® Y Data Analysis

4.8.10.1 Processing Sample Data

4.8.10.1.1 Import the sample files from a single run folder by "Edit", then selecting "Add Samples to Project".

4.8.10.1.2 In the "Add Samples to Project" screen, navigate to the run folder that contains the sample files. If the entire run folder is to be imported, click on the folder to highlight it; then click the "Add to List" button at the bottom of the window. If the run folder was shared between multiple cases, expand the folder to view the samples. Highlight the appropriate samples, ensuring that the allelic ladder and all the desired samples are selected. Once all the samples are selected click the "Add to List" button at the bottom of the window.

4.8.10.1.3 Only one injection parameter per project. A run folder **shall not** be created manually by manipulating sample files.

4.8.10.1.4 Ensure that the necessary files are now located in the "Samples to Add" window by double-clicking on the folder in the right pane, then click "Add".

4.8.10.1.5 After the samples have been added to the project, first briefly scan the raw data to ensure that a bad injection did not occur. To check the raw data, first expand the project folder in the left navigation pane, then click on a sample file, then click on the "Raw Data" tab in the right GeneMapper® window. To return to the "Samples" window, click on the project folder at the top of the left navigation pane.

4.8.10.1.6 The GeneMapper® *ID-X* project shall contain at least one allelic ladder from each run folder included in the project for proper genotyping. Multiple allelic ladders within a run folder will be averaged by the software to calculate the allelic bins. If a ladder injection is of low quality, delete the ladder or change the sample type from "Allelic Ladder" to "Sample" to remove it from consideration in calculating the bins.

4.8.10.1.7 Ensure that the table setting at the top of the screen is set to "PP16 HS".

4.8.10.1.8 In the "Sample Type" column, use the drop-down menu to select "Allelic Ladder", "Sample", "Positive Control" or "Negative Control" for each sample.

4.8.10.1.9 In the “Analysis Method” column, for each sample select “PowerPlex Y” from the drop-down menu. Click the column header cell to highlight the entire column, then select “Edit”, then “Fill Down” (or the shortcut Ctrl + D).

4.8.10.1.10 In the “Panel” column, for each sample select “PowerPlex_Y_ISP_Panels_IDX_v2.0” then choose “PowerPlex_Y_IDX_v2.0” from the drop-down menu. Click the column header cell to highlight the entire column, then select “Edit”, then “Fill Down” (or the shortcut Ctrl + D).

4.8.10.1.11 In the “Size Standard” column, select “ILS HS” from the drop-down menu. Click the column header cell to highlight the entire column, then select “Edit”, then “Fill Down” (or the shortcut Ctrl + D).

4.8.10.1.12 The Analysis Method, Size Standard, and Panel can be set as defaults when a GeneMapper® *ID-X* project is opened. Under the “File” menu, select “Project Options”. Under the “Add Samples” tab select the above settings as the default in the drop-down menus for Analysis Method, Size Standard and Panel. Click “OK”.

4.8.10.1.13 Select the green “Analyze” arrow button to start the data analysis. At the Project name prompt, save the project. At a minimum the project name shall contain the injection parameters for the project and date the sample run was started on the instrument. The case number is recommended but not required. Select the “ISP Casework Security Group”.

4.8.10.2 Evaluating Sample Data

4.8.10.2.1 The Sizing Quality shall be at least 0.75 for it to pass and should be close to 1.0.

4.8.10.2.2 Highlight all sample rows containing Allelic Ladders. Then click “View”, then “Display Plots”. In the Samples Plot screen, change the “Plot Setting” drop-down box to “PP16 HS”. Magnify the area from approximately 60 bp to 400 bp. Then click “File”>”Print”>”Print” to print off the allelic ladder electropherograms. Check the allelic ladders to ensure that the correct allele calls are made for each peak. (Refer to the PowerPlex®Y System Technical Manual for current Allelic Ladder allele calls.) Close out of the Samples Plot window.

4.8.10.2.3 Highlight all sample rows containing negative controls (ex. amplification blanks, female negative controls, and reagent blanks). Then click “View”, then “Display Plots”. In the Samples Plot screen, change the “Plot Setting” drop-down box to “PP16 HS”. Print off the entire electropherogram ensuring that the primer peak is visible by clicking “File”>”Print”>”Print” to print off the negative control electropherograms. Check the negative controls

to ensure that no peaks above threshold are present. Close out of the Samples Plot window.

4.8.10.2.4 Highlight all remaining sample rows. Then click "View", then "Display Plots". In the Samples Plot screen, change the "Plot Setting" drop-down box to "PP16 HS". Magnify the area from approximately 60 bp to 400 bp. After evaluating all allele calls, click "File">"Print">"Print" to print off all sample electropherograms. Optionally, the remaining sample rows may be viewed, evaluated, and printed with the Allelic Ladders.

4.8.10.2.5 After all analysis is complete, save the 3130 Data Collection Run Folder and associated GeneMapper® *ID-X* projects under each associated laboratory case number and request folder located in the analysts' folders on the server. Projects should be deleted monthly from the "GeneMapper® *ID-X* Manager" to maintain database space.

4.8.10.2.5.1 When exporting the GeneMapper® *ID-X* project, ensure that the "Export with analysis settings" box is checked.

4.8.10.2.6 The number of audit records on the GeneMapper® *ID-X* database should be routinely checked. Audit records should be backed-up, saved to the DNA server, and then deleted from the GeneMapper® *ID-X* database monthly or if the number of records exceeds 40,000. If the number of audit records exceeds 60,000, the performance of the software may be affected.

4.8.10.2.7 The amount of database space in the GeneMapper® *ID-X* software should be routinely checked. If the occupied space exceeds 80%, additional disk space should be allocated.

4.8.11 Interpretation Guidelines For PowerPlex® Y

4.8.11.1 Once a determination has been made whether a peak is to be considered a true allele, the following interpretation guidelines shall be used. The minimum peak height threshold is established at 50 relative fluorescent units (RFU) for GeneMapper® *ID-X* software. The analytical threshold for data interpretation is 50 RFU. The stochastic threshold for data interpretation of the DYS385 a/b locus is 250 RFU.

4.8.11.2 Peaks below 50 RFU shall not be interpreted or marked on the STR summary sheet.

4.8.11.3 Interpretation of peaks with RFU from 50 to 250 RFU at the DYS385 a/b locus is a qualitative assessment. It shall be interpreted with care. If the DYS385 a/b locus demonstrates a single allele that is above 50 RFU and below 250 RFU, true homozygosity cannot be determined due to possible stochastic effects and it will be reported with a bracket (ex. [13]) on the Y-STR summary sheet. If the DYS385 a/b locus demonstrates two alleles in which one or both alleles are above 50 RFU and below 250 RFU, and the sample appears to be single source, the allele(s) below 250 RFU will be reported with a bracket. Both alleles are

interpretable and may be used in a Y-STR database search with documented approval from the Supervisor and notification to the Technical Leader.

4.8.11.4 Peaks at the remaining loci with RFU of 50 and above are reportable based on both qualitative and quantitative assessment of data.

4.8.11.5 **Controls**:

4.8.11.5.1 The appearance of pull-up or known artifact peaks does not render the following controls inconclusive.

4.8.11.5.2 **Reagent Blank**: The purpose of the reagent blank is to determine if the reagents used to extract the associated samples were contaminated by male DNA. Therefore no signal should be detected in this sample well other than the internal lane standard.

4.8.11.5.2.1 A reagent blank with peaks below 50 RFU shall not prevent associated samples from being interpreted, but the Technical Leader and Supervisor shall be notified (with documentation in the case record) if more than two loci demonstrate peaks.

4.8.11.5.2.2 A reagent blank with peaks of 50 RFU and above shall be considered a failed negative control. All associated samples shall be inconclusive. All the samples shall be repeated when reasonable and appropriate.

4.8.11.5.3 **Positive Control**:

4.8.11.5.3.1 The 9948, 2800M or other appropriate male DNA standard supplied with the PowerPlex® Y kits is used as a positive control to demonstrate that the kit is performing properly. If the expected alleles are not detected in the positive control well, then the test is considered inconclusive.

Y-STR Locus	9948	2800M
DYS391	10	10
DYS389 I	13	14
DYS439	12	12
DYS389 II	31	31
DYS438	11	9
DYS437	15	14
DYS19	14	14
DYS392	13	13
DYS393	13	13
DYS390	24	24
DYS385 a/b	11,14	13,16

4.8.11.5.4 **Amplification blank and Female Negative control**:

4.8.11.5.4.1 The purpose of the amplification blank is to determine if male DNA contaminated the samples at the amplification step.

Because no template DNA was placed in the reaction tube, the sample well should be blank except for the internal lane standard peaks. If amplified product is detected in the amplification blank well, the test is considered inconclusive.

4.8.11.5.4.2 The purpose of the female negative control is to confirm the male specificity of the reaction. Because the negative control is female DNA, the sample well should be blank except for the internal lane standard peaks. If amplified product is detected in the negative control well, the test is considered inconclusive.

4.8.11.5.4.3 An amplification blank or a female negative control with peaks below 50 RFU shall not prevent associated samples from being interpreted, but the Technical Leader and Supervisor shall be notified (with documentation in the case record if more than two loci demonstrate peaks.)

4.8.11.5.4.4 An amplification blank or a female negative control with peaks of 50 RFU and above shall be considered failed negative controls. All associated samples shall be inconclusive. All the samples shall be repeated when reasonable and appropriate.

4.8.11.6 The analytical threshold shall be determined during validation. If an analyst has determined that a peak that has been labeled by the GeneMapper® *ID-X* software is not a true allele peak, the analyst can delete the allele call label in either the software file or manually on the printed electropherograms. The GeneMapper® *ID-X* software is set to display all allele changes. Therefore, any change or deletion in an allele call shall be visible on the electropherogram print-out.

4.8.11.7 An analyst is required to visually confirm all allelic ladders used for allele designation and the allele calls for all positive controls.

4.8.11.8 **Stutter peaks** are artifacts of the amplification process. These peaks will typically be observed in the n-4 position of major peaks for tetranucleotide repeat loci, in the n-5 position of major peaks for the pentanucleotide repeat locus, or in the n-3 position of the major peaks for the trinucleotide repeat locus. The peak heights of stutter peaks will be less intense than that of the major peak. The average observed percent stutter for each locus is listed in the table below. The mean stutter value for each locus is used as the stutter cut-off value in the marker stutter file “PowerPlex_Y_ISP_Stutter_IDX_v2.0” for GeneMapper® *ID-X* analysis. Therefore, any peaks in the n-4 (for tetranucleotide repeats), the n-3 (for trinucleotide repeats), or n-5 (for pentanucleotide repeats) stutter positions that are below these values when compared to the major peak will be automatically filtered out by the software and will not be labeled. However, these values may vary slightly. There are also stutter-like peaks at the n-9/n-10, n-5 and n+/-2 positions and are also listed on the table below. The table also lists

the mean +3 Standard Deviations (SD) value (99.7% confidence level) of stutter observed in validation. This column may be used as a guideline to the analyst for determining stutter peaks that were not filtered out by the software. It is the analyst's discretion to determine which allele calls may be renamed as stutter in GeneMapper® *ID-X* analysis. For samples which have been over-loaded, the percent stutter calculation will not be accurate due to the saturation effect of the major peak. Any peak that is in the stutter position but is questionable as to whether it is stutter or a true allele shall by marked with "^" (ex. 17^) on the Y-STR summary sheet and on the electropherogram print-out. In addition to stutter peaks, several other stutter-like peaks can be observed at some PowerPlex® Y loci.

4.8.11.8.1 **Note:** Stutter peaks have also been reported at the n+4, n+ 5, n+3, and n+6 positions as well as the n-8, n-10, and n-6 positions. These peaks will also have significantly less intense signal than the major peak and those values are listed in the table below. Other artifacts of less intensity have been reported which may not line up with the ladder. The interpretation of these peaks, similar to the other artifact peaks, shall be at the discretion of the analyst based on their training and experience.

Locus	Stutter	Mean% Stutter	Mean+3SD
DYS391	n-4	6.1	8.8
	n+4	0.4	0.9
	n-8	0.3	0.6
DYS389I	n-4	5.7	9.3
	n+4	0.4	1.8
	n-7/n-8	2.5	3.9
DYS439	n-4	5.9	10.7
	n+4	1.1	2.6
	n-8	0.4	0.9
DSY389II	n-4	12.3	16.3
	n+4	1.4	3.1
	n-8	3.2	4.5
DYS438	n-5	2.4	4.6
	n+5	0.4	0.8
	n-10	0.2	0.7
DYS437	n-4	4.6	8.0
	n+4	0.8	3.0
	n-8	1.6	3.2

	n-9/n-10	1.3	2.2
DYS19	n-4	6.1	8.8
	n+4	0.6	1.0
	n-8	0.4	0.8
	n-2	7.0	13.1
	n+2	1.1	2.1
DYS392	n-3	8.7	14.7
	n+3	4.2	8.0
	n-6	1.2	3.4
	n+6	0.4	1.0
DYS393	n-4	9.1	11.8
	n+4	2.1	4.1
	n-8	0.9	2.8
	n-9/n-10	0.7	2.2
DYS390	n-4	7.5	11.2
	n+4	0.8	3.2
	n-8/n-9	2.1	4.0
DYS385	n-4	8.2	13.4
	n+4	1.2	3.1
	n-8	0.8	2.3
	n-9/n-10	5.6	8.4

4.8.11.9 **Artifacts** have been observed utilizing the PowerPlex® Y amplification kit. The intensity of these peaks is directly related to signal intensity; therefore reducing the signal intensity to under 3000 RFU should eliminate the appearance of these types of artifacts. If an analyst renames the allele call of any artifact in GeneMapper® *ID-X*, it shall be labeled appropriately.

4.8.11.10 **Pull-up or bleed through** peaks can occur if signal intensity of sample or ILS peaks is too high or if a new spectral calibration needs to be run. Any pull-up peaks called as alleles by the GeneMapper® *ID-X* software should be labeled on the electropherogram as pull-up. The sample should be re-run if a pull-up peak interferes with the analyst's ability to evaluate the profile based on their experience and training.

4.8.11.11 **Spikes** are peaks that generally appear in all colors and are sharper than regular peaks; however, they can occur predominantly in one color. Spikes are a natural consequence of capillary electrophoresis

and can be caused by dust present in the system as well as urea crystals in the system. It is essential that the instrumentation be maintained and cleaned regularly to minimize the appearance of spikes. All spikes called as alleles by the GeneMapper® *ID-X* software should be clearly labeled as spikes on the electropherogram printout. A sample should be re-injected when a spike interferes with the analyst's ability to evaluate the profile based on their experience and training.

4.8.11.12 Rare variants have been described in the literature. The causes of these rare variants are microvariants or chromosomal mutations (duplication or deletion).

4.8.11.12.1 Microvariants: alleles one, two or three nucleotides shorter than the common four base repeat alleles (one or two nucleotides shorter in the case of three base repeat alleles or four nucleotides shorter in the case of five base repeat alleles) which are located between two alleles on the ladder shall be described as the short repeat followed by the number of base pairs it is larger (a 0.1, 0.2, 0.3, or 0.4 in the case of a pentanucleotide repeat). Therefore, if a peak is 1 base pair larger than the 5 allele it shall be designated as 5.1. The precision of sizing at a 99.7% confidence level is less than 0.25 bp which is precise enough to be confident in the sizing of microvariants. A microvariant 4 base pairs larger than an allele (or 5 base pairs for a pentanucleotide) on the ladder may be designated with the full repeat number (A peak 4 base pairs larger than the 5 allele could be designated a 6; 5 base pairs larger a 6.1).

4.8.11.12.2 Alleles which are located outside the range of the ladder or bin set (above or below) shall be described as "<" or ">" the largest or smallest allele for that locus with a set of ( ) placed around the off ladder allele. For example, if a band is located above the largest allele for the DYS19 locus, it would be designated as "(>19)". This should be clear when used and can be verified with a locus review of the electropherogram.

4.8.11.12.3 A duplication occurs when multiple alleles are present at a locus/loci. Loci at which documented duplications exist include the following: DYS19, DYS389 I, DYS389 II, DYS390, DYS391, DYS392, DYS393, and DYS385 a/b. Most duplications have a single repeat difference.

4.8.11.12.3.1 Steps to distinguish between a mixture and a duplication: determine the number of loci containing more than one peak; consider the position on the Y-chromosome if multiple loci have two peaks; determine if the repeat spread is >1 repeat unit; examine DYS385 a/b for the presence of >2 peaks; and consider autosomal testing to confirm single-source sample.

4.8.11.12.4 A deletion occurs when there is a lack of a peak at a single locus/loci.

4.8.11.13 **Mixed DNA Samples**. All loci shall be taken into consideration when interpreting a mixture. A two-peak pattern at one or more loci (except for DYS385 a/b; this locus may exhibit three or more peaks) is an indication of a mixture. However, two-peak patterns have been reported for single-source stains, but these instances are extremely rare. If a two-peak pattern is observed for a single-source questioned stain that does not match the standards submitted, and is believed to be a rare variant, the profile should be re-amplified and re-analyzed to confirm the profile. For loci in which a mixture is indicated, peak height ratios can be analyzed to assist in clarification of the major contributor of the mixture. If the peak height ratio to a lesser peak is less than 25%, a major may be called at that locus. A ">" can be used on the STR summary sheet to identify these differences in peak heights.

4.8.11.14 The "phenotype" of each profile shall be recorded on the Y-STR Summary Worksheet.

4.8.11.15 During interpretation, the analyst and technical reviewer shall each compare all unknown profiles to available staff profiles to ensure that samples have not been contaminated. All instances of profiles consistent with a staff member shall be reported to the Technical Leader.

4.8.11.16 There are four possible outcomes of Y-STR analysis:

4.8.11.16.1 No results: No peaks were detected on the electropherogram.

4.8.11.16.2 Inconclusive: Peaks were observed at one or more loci; however no conclusive results can be drawn from them.

4.8.11.16.3 Inclusion: The profile obtained from the questioned stain had no discrepant alleles as compared to the profile of the known standard. Allelic drop-out may occur in low concentrations or mixtures.

4.8.11.16.4 Exclusion: The profile obtained from the questioned stain was not the same as the known material.

4.8.12 General Rules For PowerPlex® Y Analysis On The Applied Biosystems 3130 or 3130xl Genetic Analyzer

4.8.12.1 It is required that at least one allelic ladder is present within a run folder. However, it is recommended that an allelic ladder is run within every 32 samples on a sixteen capillary instrument or within every 16 samples on a four capillary instrument. External environmental factors during a plate run can cause a shift in the migration of DNA fragments within the capillary which may cause a small difference in the base pair length determined for an allele. If an allelic ladder sample was not run

within a reasonable time as the sample, this may cause an allele to be called off ladder.

4.8.12.2 If a sample is to be re-injected at higher injection parameters, the reagent blank and the amplification blank associated with that sample shall also be re-injected at the higher parameters. The positive control need not be injected at the same parameters as the samples associated with it.

4.8.12.3 If a selection of samples from one amplification requires re-injection at higher injection parameters with their associated blanks (as above) and the blanks at the higher parameters demonstrate some indication of contamination, The Technical Leader shall be informed. The decision to declare all sample data inconclusive or just the sample data from the higher injection parameter shall be at the discretion of the analyst with the approval of the Technical Leader, documented in the case record.

4.8.12.4 Only the injection(s) used for interpretation need to be printed for the case record. However, other injection runs have to be noted in the case record and all data shall be saved under each associated laboratory case number and request folder located in the analysts' folders on the server. If individual samples in a case use different injection parameters, it shall be noted in the case record which injection was used for interpretation for each sample.

4.8.12.5 The placement of unknown samples in the 96-well plate should be done so that the orientation allows for the injection of unknown samples prior to the injection of any standards for that case.

4.8.12.6 If multiple analysts are sharing a 96-well plate, two empty columns of wells should be maintained between each analyst's samples to allow for separate preparation and loading of samples. The plate septa can be placed on the plate prior to the addition of samples. The septa can be cut down the appropriate column containing the empty wells with a scalpel allowing wells to remain covered while the other analyst is preparing their portion of the plate.

4.8.12.7 Peak heights of analyzed samples shall not exceed 7,000 RFU. This level of peak height is approaching saturation and will lead to the appearance of artifacts. Reducing the signal to approximately 1,000 - 2,000 RFU will produce optimal results. The reduction of signal can be obtained by decreasing the injection parameters. The allowable injection parameters are 3kV 5 second or 3 kV 3 second. Use of data with a single peak >7,000 RFU may be allowed with the approval of a Supervisor and notification to the Technical Leader documented in the case record.

4.8.13 Archiving Applied Biosystems 3130 or 3130XL and GeneMapper® *ID-X* Projects

4.8.13.1 The 3130 or 3130XL run folders containing all sample files for a case as well as the GeneMapper® *ID-X* project file shall be saved under each associated laboratory case number and request folder located in the analysts' folders on the server and deleted from the hard drive of the instrument and/or analysis computer. The data stored on the server shall be routinely backed up to ensure security of data.

4.8.13.2 A hard copy of all electropherograms used in interpretation as well as a print-out of the plate record shall be placed in the case record.

4.8.13.3 All processed plate records shall be deleted from the Data Collection Software database weekly. Run folders containing the sample files and the GeneMapper® *ID-X* projects shall be deleted on or after the 15th of each month on the instrument computers. It shall be each analyst's responsibility to ensure that all data is backed up prior to the 15th of the month.

4.8.14 Mixture Interpretation

4.8.14.1 A profile is defined as a mixture between two or more males when two or more loci demonstrate two or more alleles. A profile where only one locus demonstrates two alleles could be an indication of:

4.8.14.1.1 A mixture where the minor contributor is not at levels allowing interpretation;

4.8.14.1.2 Extraneous DNA;

4.8.14.1.3 A duplication.

4.8.14.2 In situations 1 and 2 above, report wording to the affect that one lone allele was detected and no conclusion can be reached is appropriate. In situation 3 no mention is required in the Certificate of Analysis.

4.8.14.3 If the profile is determined to be a mixture, each locus should be evaluated and classified as below:

4.8.14.3.1 Indistinguishable mixture between two or more males; in this case, since no statistical interpretations can be made, inclusions will not be made for these samples.

4.8.14.3.2 Mixture demonstrating a major contributor

4.8.14.3.3 Demonstrates the potential for allelic drop-out.

4.8.14.4 A profile demonstrating six or more loci classified as a mixture demonstrating major and minor components shall have a major single source profile calculated based on the following criteria:

4.8.14.4.1 If the peak height ratio between the peaks is less than 25%, then the highest peak can be called a major at that locus.

4.8.14.4.2 If there is only one peak at a particular locus (and a major can be determined at one or more loci with more than one peak), this peak can be called a major. All loci must be considered in making this determination.

4.8.14.4.3 If the sample does not meet these criteria, do not call a major at this locus.

4.8.15 Y-STR Database Searching

4.8.15.1 A Y-STR profile is treated as a single locus because it is a haplotype.

4.8.15.2 Once a comparison between a reference sample(s) and a questioned sample is completed and an inclusion for a single source or major Y-STR profile shall be reported, the significance of that inclusion is estimated. It shall be reported that all paternally related males will share the same Y-STR profile.

4.8.15.3 The questioned haplotype developed for the evidence profile shall be searched against the U.S. Y-STR Database, found online at www.usystrdatabase.org.

4.8.15.4 The number of occurrences (matching samples) found in the database from the search (counting method) shall be included in the Certificate of Analysis as a frequency estimate with the application of the 95% confidence interval. This shall also acknowledge that the search results given were accurate on the date of the search.

4.9 Records

4.9.1 The appropriate worksheets or the equivalent workbooks shall be used to record all procedures. These will be found in the Worksheet Manual.

4.9.2 All data sheets, notes and other information generated from the laboratory examination shall be kept in the case record.

4.9.3 The technical review of the case record shall be recorded on the technical review worksheet.

4.9.4 Electronic records shall be retained as indicated in Appendix 5.

4.10 Interpretation of Results

4.10.1 When test results from a question sample are compared with a known standard the following conclusions may be reported:

4.10.1.1 The question sample and the known standard may have common origin. If this is indicated, it shall be reported that all paternally related males will share the same profile and a frequency of occurrence in the U. S. Y-STR Database shall be calculated and reported out at a 95% confidence interval.

4.10.1.2 The known standard may be excluded as a possible source of the questioned sample.

4.10.1.3 No conclusion may be reached with regard to the question sample and the known standard.

4.10.2 If test results from a sample cannot be clearly interpreted, the results shall be reported as inconclusive.

4.10.3 See Interpretation guidelines in 4.8.12, 4.8.15, and 4.8.16.

4.11 Wording of Y-STR Analysis Results/Opinions/Interpretations (required) {options} [example]

Because the markers will not be listed in the body of the Y-STR analysis report, the following introductory statement shall precede the Y-STR analysis section of a report:

> In the DNA analysis detailed below, the following Y-STR loci were analyzed by Polymerase Chain Reaction (PCR): DYS391, DYS389 I, DYS439, DYS389 II, DYS438, DYS437, DYS19, DYS392, DYS393, DYS390, and DYS385 a/b.

General Results:

Full Profile (No Standards For Comparison):

> A Y-STR DNA profile was obtained from X (item #).
>
> A mixed Y-STR DNA result was obtained from X (item #).
>
> A mixed Y-STR DNA result, which demonstrates a major component, was obtained from X (item #).

Limited Or No Profile:

> The Y-STR DNA profile obtained from X (item #) failed to demonstrate conclusive results.
>
> X (item #) failed to demonstrate a Y-STR DNA profile.

Use Of Exclusionary Statement (Full Profile Obtained From Sample; Standard Is Excluded):

> The Y-STR DNA profile obtained from X (item #) is consistent with an unknown male. John Doe (item #) can be excluded as a contributor to the sample.

Use Of Consistent Statement (Full Profile Obtained From Sample; No Differences From Standard):

> The Y-STR DNA profile obtained from X (item #) is consistent with the Y-STR DNA profile obtained from John Doe (item #). Therefore, John Doe and all his male paternal relatives cannot be excluded as potential Y-STR DNA contributors to the sample. Utilizing a published Y-STR DNA population database, the Y-STR profile has been observed as follows:
>
> ___ in ____ Caucasian individuals. Applying the 95% upper confidence interval results in approximately 1 in every _____ individuals.
> ___ in ____ African American individuals. Applying the 95% upper confidence interval results in approximately 1 in every _____ individuals.

___ in ____ Hispanic individuals. Applying the 95% upper confidence interval results in approximately 1 in every _____ individuals.

The following loci were used in the above statistical calculation: (list loci). The above statistics were calculated using the U.S. Y-STR Database (http://www.usystrdatabase.org) on [January 1, 2011].

(Note: statistic sheets will be printed out for every sample)

Partial Profile Obtained from Sample:

The partial Y-STR DNA profile obtained from X (item #) is consistent with the Y-STR DNA profile obtained from John Doe (item #). Therefore, John Doe and all his male paternal relatives cannot be excluded as potential Y-STR DNA contributors to the sample. Utilizing a published Y-STR DNA population database, the partial Y-STR profile has been observed as follows:

___ in ____ Caucasian individuals. Applying the 95% upper confidence interval results in approximately 1 in every _____ individuals.
___ in ____ African American individuals. Applying the 95% upper confidence interval results in approximately 1 in every _____ individuals.
___ in ____ Hispanic individuals. Applying the 95% upper confidence interval results in approximately 1 in every _____ individuals.

The following loci were used in the above statistical calculation: (list loci). The above statistics were calculated using the U.S. Y-STR Database (http://www.usystrdatabase.org) on [January 1, 2011].

Mixtures (A Profile With A Two Peak Pattern At One Or More Loci; Excluding DYS385):

Indistinguishable Mixture:

The Y-STR DNA result obtained from X (item #) demonstrated the presence of a mixture of at least two male individuals. The results cause an inability to provide statistical calculations; therefore, no conclusions will be provided regarding John Doe (item #) as being a possible contributor to the mixture.

Distinguishable Major And Minor Components:

The Y-STR DNA result obtained from X (item #) demonstrated the presence of a mixture with a major DNA profile. The major Y-STR DNA profile is consistent with John Doe (item #). Therefore, John Doe and all his male paternal relatives cannot be excluded as potential Y-STR DNA contributors to the sample. No conclusion can be drawn from the remaining alleles. Utilizing a published Y-STR DNA population database, the major Y-STR profile has been observed as follows:

___ in ____ Caucasian individuals. Applying the 95% upper confidence interval results in approximately 1 in every _____ individuals.
___ in ____ African American individuals. Applying the 95% upper confidence interval results in approximately 1 in every _____ individuals.
___ in ____ Hispanic Individuals. Applying the 95% upper confidence interval results in approximately 1 in every _____ individuals.

The following loci were used in the above statistical calculation: (list loci). The above statistics were calculated using the U.S. Y-STR Database (http://www.usystrdatabase.org) on [January 1, 2011].

Where Examination Of Samples Within A Subitem Is Not Performed:

No Y-STR analysis was performed on the [number] additional samples within item # at this time.

Where Evaluation Of The Case Does Not Support Performing Y-Str Analysis:

This case was evaluated for possible Y-STR analysis. No samples were found to be suitable for Y-STR analysis.

Request For Standards:

Additional Y-STR DNA comparisons will be made upon submission of an appropriate male DNA standard(s) (such as a blood standard in a purple top tube or an oral swab standard).

Sample Wording For Database Entry (This Paragraph Shall Be At The End Of Any Report Detailing New Profiles).

At this time, Y-STR samples cannot be entered or searched in the Indiana DNA Database.

Sample Wording of Retention Statement

All subitems created from originally submitted items will be retained by the Indiana State Police Laboratory for the possibility of future analysis.

4.12 References

4.12.1 Promega Corporation, (2012 or most current version). Plexor® HY System for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems Technical Manual. Part No. TM293. Madison, WI.

4.12.2 Krenke, B.E. The Plexor™ Analysis Software. Profiles in DNA. 2005. 8(2): pp.6 – 7.

4.12.3 Promega Corporation, (2008 or most current version). PowerPlex® Y System Technical Manual. Part No.TMD018. Madison, WI.

4.12.4 Butler, J.M. 2010. Lineage Markers: Y Chromosome and mtDNA testing. Fundamentals of Forensic DNA Typing. pp 363-374. Academic Press. Burlington, MA.

4.12.5 Butler, J.M. 2005. Y Chromosome DNA Testing. Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers. Second Edition. pp 201-239. Elsevier Academic Press. Burlington, MA.

4.12.6 Ballantyne, J. and Fatolitis, L. The U.S. Y-STR database. Profiles in DNA. 2008. 11(1): 13 – 14.

4.12.7 U.S. Y-STR Database. http://usystrdatabase.org.

5. DNA Sample Processing using the Biomek NX^P and Biomek 3000 Automated Workstations:

5.1. Scope:

5.1.1. This test method is designed for the direction of laboratory personnel who will operate the Biomek NX^P and Biomek 3000 automation workstations to process DNA samples from extraction/purification through CE (capillary electrophoresis) setup. The Biomek NX^P workstation is capable of extracting up to 79 samples and blanks in two hours, setting up quantification plates, normalizing samples and setting up amplification. The Biomek 3000 workstation then transfers amplified product to a CE plate to be run on a genetic analyzer. This test method may be expanded or altered as techniques and/or new genetic analyses are found applicable and validated.

5.2. Precautions/Limitations:

5.2.1. Due to the hands-off nature of automated processing, it is vital that submitted samples be prepared following procedures determined by the automation validation and that the ordered flow of data throughout the process be maintained.

5.2.2. Customization or modification of sample processing procedures will not be allowed without consultation with the automation team and documented approval of the Technical Leader.

5.3. Related Information:

5.3.1. N/A

5.4. Instruments:

5.4.1. Biomek NX^P Automated Laboratory Workstation— robotic DNA processing system.

5.4.2. Biomek 3000— robotic DNA processing system.

5.5. Reagents/Materials

5.5.1. Digest/Wash Buffer
5.5.2. Isopropyl Alcohol
5.5.3. Nuclease Free Water (NFH_2O)
5.5.4. DTT 1M
5.5.5. Proteinase K (10 mg/ml)
5.5.6. Proteinase K (18 mg/ml)
5.5.7. Ethanol
5.5.8. Sarkosyl 20% w/v
5.5.9. Stain Extraction Buffer for Automation
5.5.10. TRIS/EDTA/NaCl Solution
5.5.11. DNA IQ Extraction Kit

5.5.11.1. Lysis Buffer
5.5.11.2. Elution Buffer
5.5.11.3. Magnetic Resin
5.5.11.4. Wash Buffer

5.6. Hazards/Safety

5.6.1. All chemicals shall be handled in a safe method as referenced in the specific Material Safety Data Sheets (MSDS).

5.6.2. Universal Precautions shall be observed whenever biological materials are being handled.

5.6.3. Biological waste shall be disposed of in the appropriate waste receptacle.

5.6.4. On the Biomek NX^{P}, a light curtain prevents interaction with the deck or the robotic arm while a procedure is running.

5.6.5. There is an emergency stop button on the front of the Biomek 3000.

5.7. Reference Materials/Calibration Checks

5.7.1. DNA processing using the automated workstation does not require any additional controls to those used in manual DNA processing.

5.7.2. Annual calibration of the pipetting accuracy will be performed by Beckman Coulter Operational Qualification 3 (OQ3).

5.8. Procedures/Instructions

5.8.1. General Rules

5.8.1.1. Batch ID numbers shall be named sequentially “year-run number” (ex. 2011-001).

5.8.1.2. Each case shall contain at least one reagent blank for unknowns when processed on the automated workstation.

5.8.1.3. Each group of standards/knowns (per analyst) processed on the automated workstation shall contain at least one reagent blank. On the pre-extraction worksheet (submission form), the case number for the grouped reagent blank shall be the first case number in the batch it is associated with.

5.8.1.4. Reagent blanks shall contain “RB” in the name on the pre-extraction worksheet.

5.8.1.5. Within 24 hours of incubation, analysts shall deliver samples to be processed to the automation room. An e-mail shall be sent to the automation team at DAutomation@ISP.IN.gov with the completed pre-extraction worksheet attached. The form shall be named

"date_initialsPE_submission#" (ex. 01Jan11_AB1234_01) and the tube rack containing the samples shall be labeled the same.

5.8.1.6. To maximize efficiency of each run, the automation team may process samples up to 14 days from their submission date.

5.8.1.7. When entered on the pre-extraction worksheet, case numbers and sample names shall not contain special characters except for dashes and underscores.

5.8.1.8. In addition to the documentation of data as described in the Forensic Biology Section Test Method (DNA Test Methods, section 2), the associated automation documents to be printed and retained in the case record are the pre-extraction worksheet, the extraction/quantification plate record, the amplification/capillary electrophoresis plate record, and the amplification normalization report.

5.8.2. Sample Preparation (see sampling protocols with Organic extraction)

5.8.2.1. Regular Extraction

5.8.2.1.1. Prepare Buffer/Pro K/DTT Master Mix

5.8.2.1.1.1. For each sample, combine 350 µl Stain Extraction Buffer for Automation with 10 µl Pro K (18 mg/ml) and 40 µl DTT (1M).

5.8.2.1.1.1.1. *Example: for 16 samples, combine 5600 µl Buffer with 160 µl Pro K and 640 µl DTT.*

5.8.2.1.2. Place sample at the bottom of a labeled 1.5 ml microcentrifuge tube and add 400 µl Buffer/Pro K/DTT Master Mix. Close the tube cap, vortex briefly and spin down.

5.8.2.1.2.1. Do NOT add more than 400 µl of liquid per sample.

5.8.2.1.2.2. For extraction on the Biomek NX^P workstation, samples shall be loaded specifically into Eppendorf (Hamburg, Germany) Safe-Lock 1.5 ml Tubes (Cat. no. 022363212). Do not use "dolphin-style" or other microcentrifuge tubes.

5.8.2.1.3. Incubate the sample at 56°C for at least one hour. Alternatively, samples may be incubated in this manner overnight.

5.8.2.1.4. Vortex sample briefly and spin down.

5.8.2.1.5. Remove cutting and proceed to 5.8.2.1.7 or:

5.8.2.1.6. Place the cutting into a spin basket in the labeled 1.5 ml microcentrifuge tube. Close the sample tube cap. Centrifuge at room temperature for at least 2 minutes at maximum speed. Carefully remove and discard the spin basket and cutting.

5.8.2.1.7. Close the lid of the sample tube and cut the hinge.

5.8.2.1.8. Save until ready for automated DNA extraction. Do not refrigerate or freeze sample. Prior to submission to the automation team, processed samples may be left at room temperature (22-25°C) overnight, if necessary.

5.8.2.2. Differential Extraction

5.8.2.2.1. Master Mix: For each sample (cutting), combine 240 µl Tris/EDTA/NaCl, 6 µl 20% Sarkosyl, 54 µl NFH_2O and 3 µl Proteinase K (10 mg/ml).

5.8.2.2.1.1. *Example: for 16 samples, combine 3840 µl Tris/EDTA/NaCl, 96 µl 20% Sarkosyl, 864 µl NFH_2O, and 48 µl Pro K.*

5.8.2.2.2. Place sample at the bottom of a labeled 1.5 ml microcentrifuge tube and add 300 µl of the Master Mix. Close the tube cap, vortex briefly and spin down.

5.8.2.2.2.1. For extraction on the Biomek NX^P workstation, samples shall be loaded specifically into Eppendorf Safe-Lock 1.5 ml Tubes. Do not use "dolphin-style" or other microcentrifuge tubes.

5.8.2.2.3. Incubate the sample at 37°C for 2 hours.

5.8.2.2.4. Vortex sample briefly and spin down.

5.8.2.2.5. Place the cutting into a spin basket in the labeled 1.5 ml microcentrifuge tube. Close the sample tube cap. Centrifuge at room temperature for at least 2 minutes at maximum speed. Carefully remove and discard the spin basket and cutting.

5.8.2.2.6. Carefully remove the supernatant to a clean, labeled 1.5 ml microcentrifuge tube. This is the non-sperm fraction.

5.8.2.2.7. Wash the sperm pellet with 500 µl of Digest/Wash Buffer and spin down for about 5 minutes. Carefully remove and discard the supernatant.

5.8.2.2.8. Repeat step 5.8.2.2.7 four times for a total of five washes. Approval from the Technical Leader, documented in the case record, is required to use less than the five washes.

5.8.2.2.9. Lysis Buffer Master Mix: For each sample pair (sperm and non-sperm fraction) combine 600 µl of Lysis Buffer with 6 µl of 1M DTT.

5.8.2.2.10. Add 400 µl of the Lysis Buffer master mix to each sperm cell fraction sample and 200 µl to each non-sperm fraction sample.

5.8.2.2.11. Vortex sample briefly and spin down. Cut the hinge of the sample tube.

5.8.2.2.12. Save until ready for automated DNA extraction. Do not refrigerate or freeze sample. Prior to submission to the automation team, processed samples may be left at room temperature (22-25°C) overnight, if necessary.

5.8.3. Biomek NX^{P} Preparation

5.8.3.1. Wipe down the deck with 70% ethanol or 10% bleach.

5.8.3.2. Ensure that the workstation trash is not full.

5.8.3.3. Ensure that the waste carboy is not full.

5.8.3.4. Ensure that there is enough distilled water in the intake carboy (the valve must be underwater).

5.8.3.5. Ensure that the software is connected to the instrument and not in Simulate mode:

5.8.3.5.1. "Instrument" menu → "Hardware Setup"

5.8.3.5.2. Set Port option to "COM1"

5.8.3.5.3. Click "Reconnect" and then "Accept."

5.8.3.6. Run "Home All Axes" under the Instrument menu.

5.8.3.6.1. Let the tips flush until there are no bubbles created when the pistons move.

5.8.3.6.2. There may be a large bubble stationary at the tops of the syringes; this is normal.

5.8.3.7. Ensure that ethanol and isopropyl alcohol have been added to the alcohol wash buffer in the DNA IQ kit.

5.8.4. Initial Tube Transfer

5.8.4.1. Place sample tubes in the tube racks (4x6 with white adapters), arranging the tubes according to the order shown in the Biomek Workbook. Do not skip columns or spaces between samples.

5.8.4.2. Ensure that the sample tube lid hinges have been cut.

5.8.4.3. Spin down the tube racks (containing the sample tubes) in the centrifuge. Carefully remove the sample tube lids and discard.

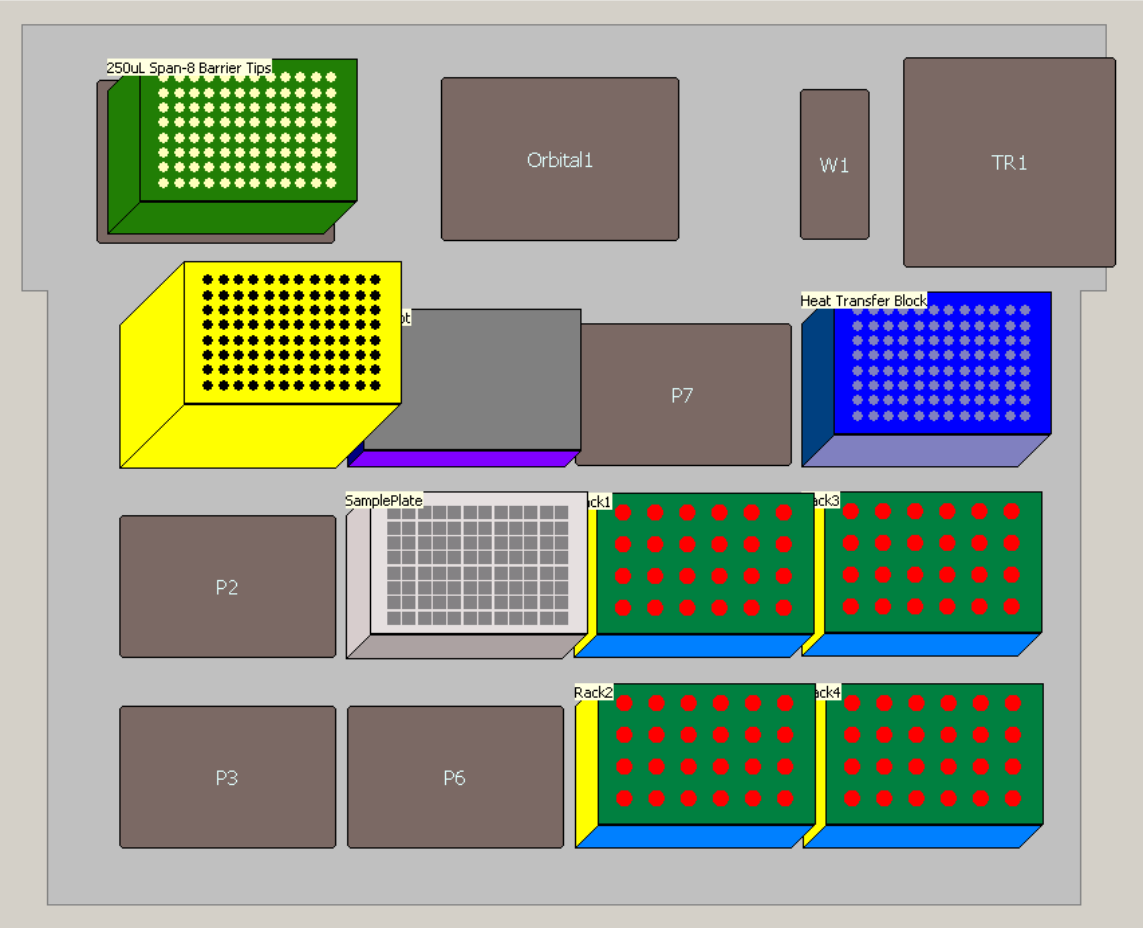
5.8.4.3.1. CAUTION: Once the lids are removed, the Biomek Workbook is the only record of the order and identity of each sample.

5.8.4.4. Arrange the deck layout as shown in the figure below:

5.8.4.4.1. Place sample tube racks on positions P8, P9, P11, and P12, again paying attention to sample order according to the Biomek Workbook.

5.8.4.4.2. Place a new Promega (Madison, WI) 2.2 ml Square-Well Deep Well Plate (Cat. no. V6781) on position P5. Orient the plate so that the clipped corner is on the bottom left.

5.8.4.4.3. Place a new box of Beckman Coulter (Fullerton, CA) Biomek® P250 Span-8 Barrier Tips (Cat. no. 379503) on the Rapid-Shuck module (top left).



5.8.4.5. Open the “Initial Tube Transfer” method contained in the Promega project.

5.8.4.6. Click the green "Run" button at the top of the screen.

5.8.4.7. The method will prompt for two values:

5.8.4.7.1. SampleColumns = the number of full or partial columns of sample on the extraction plate.

5.8.4.7.1.1. *Example: 20 samples/blanks will use two full columns and half of a third. Enter "SampleColumns" as 3.*

5.8.4.7.2. SampleVolume = the approximate volume of liquid in the sample tubes, in microliters. Round to the next highest hundred microliters, to a maximum of 400 ul.

5.8.4.7.2.1. *Example: Most of the sample tubes contain 300 ul, but one tube contains 340 ul. Enter "SampleVolume" as 400.*

5.8.4.8. When the instrument has finished transferring sample from the tubes to the sample plate, remove the tube racks from the deck and discard the empty sample tubes.

5.8.4.9. Leave the used P250 Span-8 Barrier Tips on the Rapid-Shuck module for subsequent use in DNA IQ automated extraction. Each tip is now associated with a single sample and will only be used to pipet that sample.

5.8.4.10. Remove the 2.2 ml Square-Well Deep Well Plate [containing sample transferred during Initial Tube Transfer (5.8.4)] and cover with a temporary seal. Spin the plate using either a centrifuge or a salad spinner to remove any bubbles and force samples into the bottom of each well.

5.8.5. DNA IQ™ Automated Extraction Procedure

5.8.5.1. Instrument Deck Setup

5.8.5.1.1. Turn on the heating block via the switch to the right of the Biomek NX^P, and ensure that temperature is set to 85^0C.

5.8.5.1.2. Place the 2.2 ml Square-Well Deep Well Plate sample plate [containing sample transferred during Initial Tube Transfer (5.8.4)] on position P5.

5.8.5.1.3. The box of P250 Span-8 Barrier Tips used in the Initial Tube Transfer (5.8.4) should be unchanged on the Rapid-Shuck module. DO NOT change the orientation of this box.

5.8.5.1.4. Place the following labware on the deck as shown in the figure below:

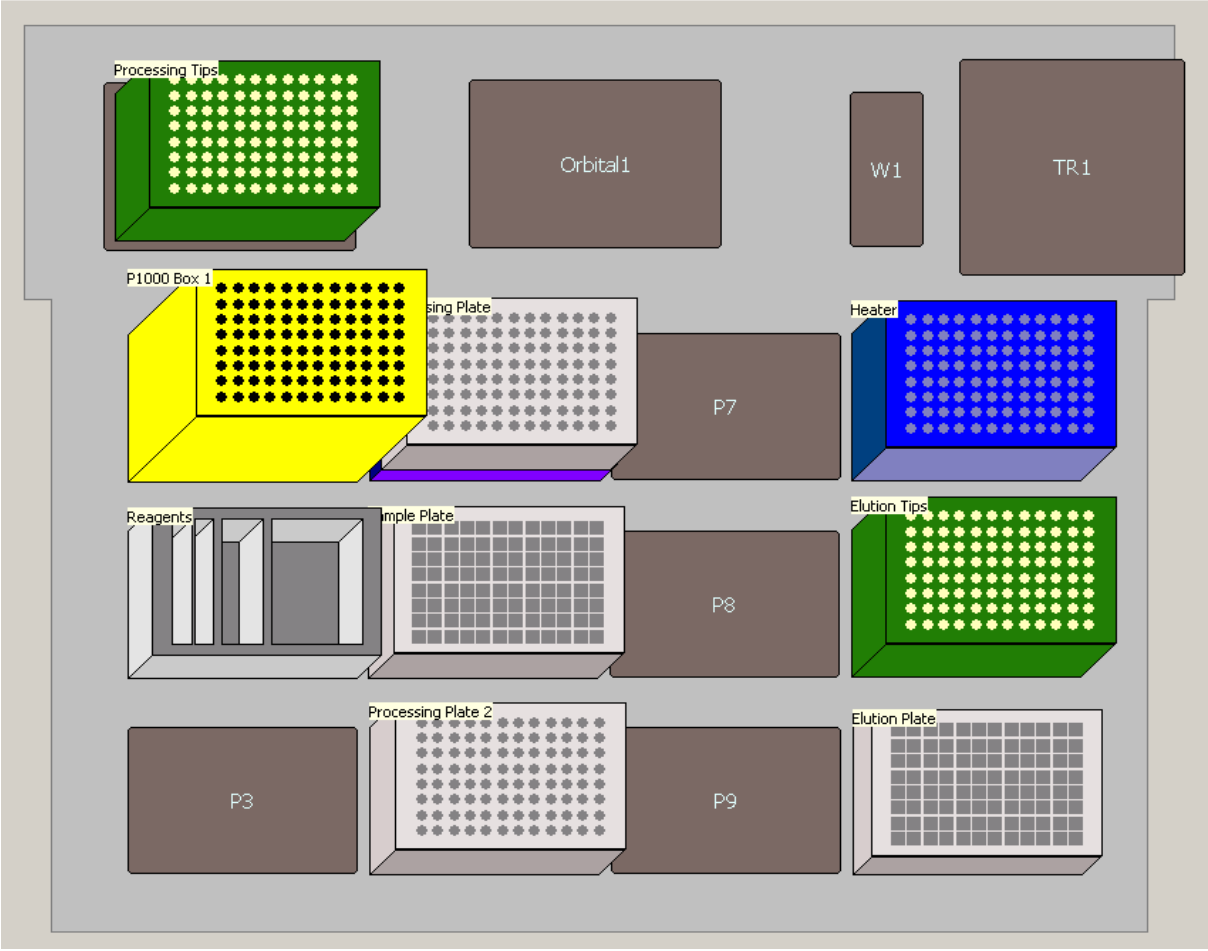
5.8.5.1.4.1. Box of Beckman Coulter Biomek® P1000 Span-8 Tips (Cat. no. 987925) on position P1. If there are fewer than five columns of

tips available, the program will prompt for a full box of P1000 tips on position P3, otherwise, a partial box may be used.

5.8.5.1.4.2. Box of new P250 Span-8 Barrier Tips on position P11.

5.8.5.1.4.3. Promega 1.2 ml Round-Bottom Deep Well Plates (Cat. no. V6771) on positions P6 and P4 (aligned on top of the MagnaBot module in P4). Orient each plate so that the clipped corner is on the bottom left.

5.8.5.1.4.4. Promega 1.1 ml Square-Well V-Bottom Deep Well Plate (Cat. no. V6821) on position P12. Orient the plate so that the clipped corner is on the bottom left.



5.8.5.2. Reagent Setup

5.8.5.2.1. Open the Biomek Workbook from the “Robot Team” network folder.

5.8.5.2.2. The “Reagents” tab has a description of the reagents and supplies needed to run automated DNA IQ extraction.

5.8.5.2.2.1. Enter the number of samples on the plate, including reagent blanks.

5.8.5.2.2.2. Enter the desired elution volume (between 50-100 µl per sample).

5.8.5.2.2.3. Do not count the No Template Control (NTC) or quantification standards.

5.8.5.2.2.4. Do not add extra samples for excess volume; it is already calculated into the program.

5.8.5.2.3. In a reservoir holder, place the following clean reservoirs in order from left to right: one Quarter Module reservoir (Beckman Coulter, Fullerton CA, cat. no. 372790) divided by height, one additional Quarter Module reservoir, and one Half Module reservoir (Beckman Coulter, cat. no 372786).

5.8.5.2.4. Load reagents in each reservoir as indicated in the “Reagents” tab, adding elution buffer last.

5.8.5.2.5. Place the reservoir holder on deck position P2.

5.8.5.3. Running the DNA IQ Method

5.8.5.3.1. Open the “DNA IQ NX Span8_v2.0.0.ISP” method, contained in the Promega project.

5.8.5.3.2. Click the “Run” button at the top of the screen.

5.8.5.3.3. The method will prompt for two values:

5.8.5.3.3.1. UsedP1000TipCols = the number of **used** full or partial columns in the 1000 µl tip box on position P1. For a new box, enter 0.

5.8.5.3.3.1.1. *Example: If two and a half columns of P1000 tips have been used, enter “UsedP1000TipCols” as 3.*

5.8.5.3.3.2. UsedP200TipCols = the number of **used** full or partial columns in the 250 µl tip box on the Rapid-Shuck module. Assuming a new box was used during the Initial Tube Transfer (5.8.4), the value should be 0.

5.8.5.3.4. A plate map interface will appear.

5.8.5.3.4.1. Enter the approximate sample volume.

5.8.5.3.4.2. Enter the desired elution volume between 50 and 100 µl (should match the elution volume entered in the Biomek NX Workbook, above).

5.8.5.3.4.3. Set Sample Type to “Aqueous”

5.8.5.3.4.4. Set “Perform Lysis Wash?” to “Yes.”

5.8.5.3.4.5. Select the wells to be processed.

5.8.5.3.4.5.1. Click on any well in a column to select the entire column.

5.8.5.3.4.5.2. Click/hold on any well and drag the cursor out of the well to select that well only.

5.8.5.3.4.5.3. Hold Ctrl to select separate wells/columns, and Shift to select consecutive wells/columns.

5.8.5.3.4.5.4. Do not select the last two columns (11 & 12). These will always be used for quantification standard.

5.8.5.3.4.5.5. Click “Enter.” The wells you selected will change color.

5.8.5.3.4.6. Step 5.8.5.3.4.5 may be repeated to specify different sample volumes or elution volumes, but doing so will change the amount of reagents required for the run (see Reagent Preparation section and step 5.8.5.3.5.1, below).

5.8.5.3.4.7. When all samples have been selected and specified, click “Finished.”

5.8.5.3.5. A map of the reagent reservoir will appear.

5.8.5.3.5.1. Unless the sample volumes have been altered, the reagent volumes should match what the Biomek NX Workbook displayed in the Reagent Preparation section, above.

5.8.5.3.5.2. Click “Close Window.”

5.8.5.3.6. The protocol will run for approximately 1 hour 45 minutes, depending on how many samples are being processed.

5.8.5.3.7. When the run is finished, reservoirs may be carefully rinsed, labeled, and reused. Discard partial tip box from position P11. Remaining partial tip boxes may be used again. Discard processing plates (both 1.2 ml round Round-Bottom Deep Well Plates). Turn off the heating block.

5.8.5.3.8. Label and retain the original sample plate (2.2 ml Square-Well Deep Well Plate) containing the lysate, as back-up until the samples have been completely processed. Cover and store at room temperature.

5.8.5.3.9. If the elution plate (1.1 ml Square-Well V-Bottom Deep Well Plate) containing the extracted samples is not to be quantified immediately, cover with a temporary seal and refrigerate.

5.8.6. REAL-TIME PCR QUANTIFICATION USING QUANTIFILER® DUO Prepared on the Biomek NX^{P} Laboratory Automation Workstation

5.8.6.1. Instrument Deck Setup

5.8.6.1.1. In the Biomek Software, open the "ABI_Quant+MM+NTC" method contained in the ABI project.

5.8.6.1.2. Click on the "User Interface" line within the method.

5.8.6.1.2.1. To the right, check that "Quantifiler Duo" is selected.

5.8.6.1.2.2. Enter the number of samples to process (equal to the number of elution plate wells that contain extract).

5.8.6.1.2.3. Do not count the NTC or quantification standards.

5.8.6.1.2.4. Do not add extra samples for excess volume; it is already calculated into the program.

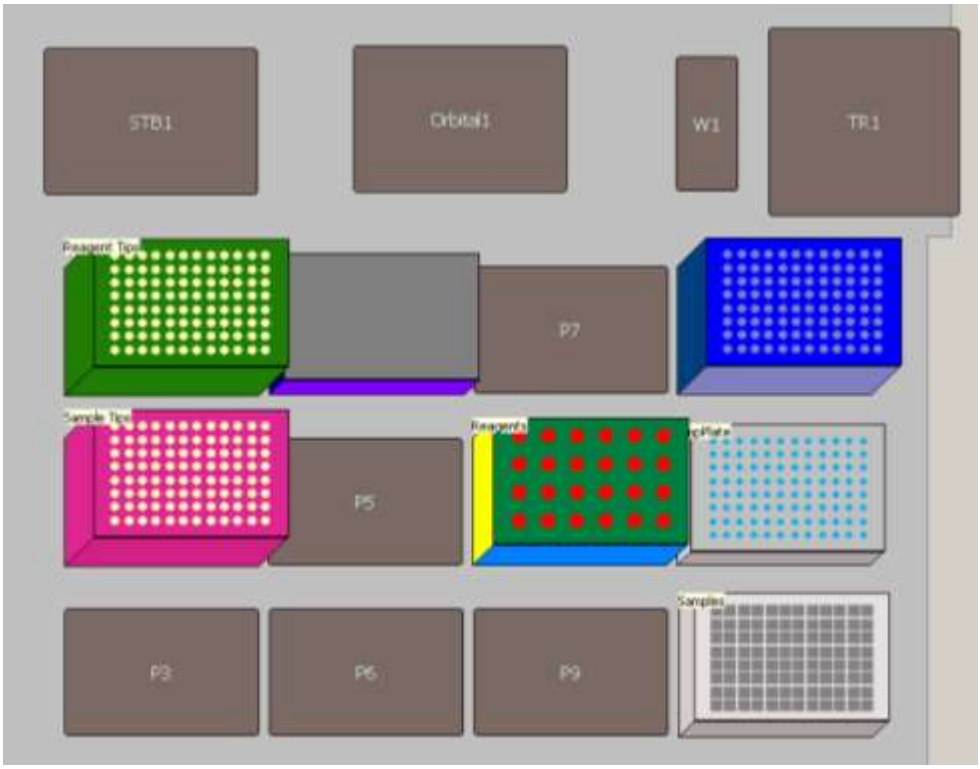
5.8.6.1.3. Place the following labware on the deck as shown in the figure below:

5.8.6.1.3.1. New box of Beckman Coulter Biomek® P50 Barrier Tips (Cat. no. A21586) on position P2. Alternatively, a partial box may be used by selecting the number and position of remaining tips in the box under the "Properties" tab of the "Instrument Setup" step.

5.8.6.1.3.2. Box of P250 Span-8 Barrier Tips on position P1. Note: The last two columns of elution tips from the DNA IQ method can be used here; turn the box around so that the tips are in the two leftmost columns.

5.8.6.1.3.3. New Applied Biosystems (Foster City, CA) MicroAmp® Optical 96-Well reaction plate (Cat. no. N801-0560) on position P11.

5.8.6.1.3.4. The elution plate containing extracted samples on position P12.



5.8.6.2. Reagent Preparation

5.8.6.2.1. Open the Biomek NX Workbook from the RobotTeam folder.

5.8.6.2.2. The “Reagents” tab has a description of the reagents and supplies needed to run automated quantification setup.

5.8.6.2.2.1. Enter the number of samples on the plate, the same as entered into the “User Interface” above.

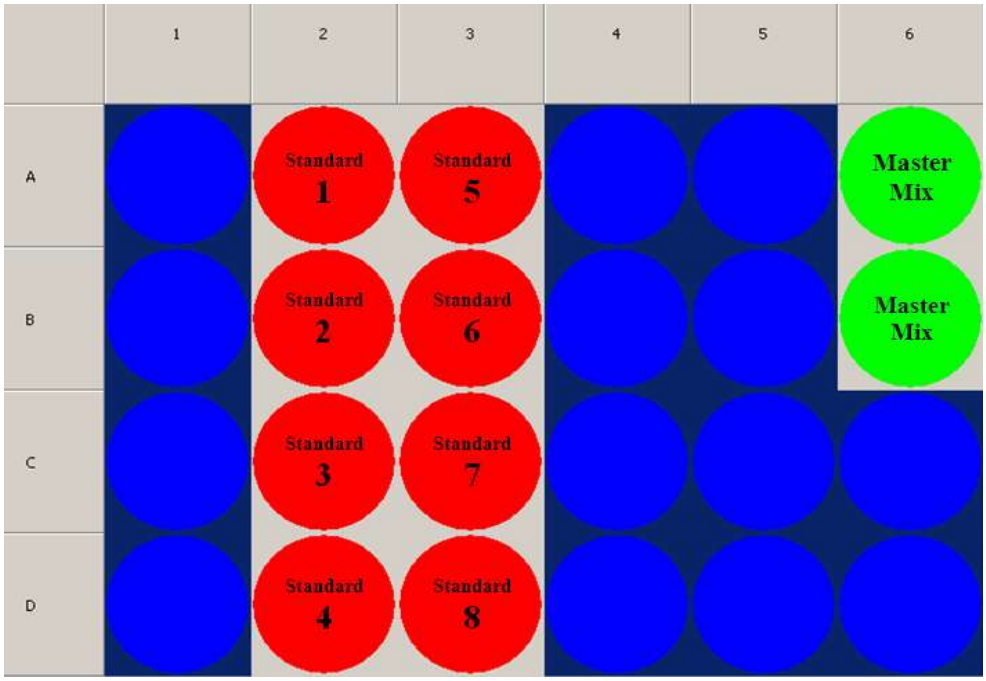
5.8.6.2.3. Prepare the master mix as described in section 2 of the Forensic Biology Section Casework Test Method, using the volumes indicated on the Reagents tab. Note that for 40 or more samples, the master mix must be divided evenly into two tubes. Vortex the Master Mix 3 to 5 seconds, then centrifuge briefly.

5.8.6.2.4. Place the master mix tube(s) in wells A6 (and B6) of the Reagent Tube Rack as indicated below.

5.8.6.2.5. Prepare the standard dilution series as described in section 2 of the Forensic Biology Section Casework Test Method, or use a previously prepared dilution series. Each tube must contain at least 20 µl of standard.

5.8.6.2.6. Place the standard tubes in columns 2 and 3 of the Reagent Tube Rack as indicated below.

5.8.6.2.7. Open and fold back all tube caps and place the Reagent Tube Rack on deck position P8.



5.8.6.3. Running the Automated Quant Duo Setup Method

5.8.6.3.1. With the “ABI_Quant+MM+NTC” method open and the number of samples specified, click the “Run” button at the top of the screen.

5.8.6.3.2. A window will appear to confirm the amount and placement of the master mix tubes. Click “OK.”

5.8.6.3.3. A window will appear to confirm the deck layout as shown by the software. Click “OK.”

5.8.6.3.4. A window will appear to confirm the total amount of master mix, the amount of standard in each of the standard tubes, the sample count, and the quantification kit. Click “OK.”

5.8.6.3.5. The protocol will begin. When the run is finished, remove the elution plate with extracts and cover with a temporary seal until ready to proceed with amplification.

5.8.6.3.6. Seal the optical plate with an optical adhesive cover. Run the edge of the cover applicator between the rows and columns of the wells to ensure that all wells are sealed properly.

5.8.6.3.7. Spin the plate using either a centrifuge or a salad spinner to remove any bubbles and force samples into the bottom of each well.

5.8.6.3.8. Place the plate in the real-time PCR instrument, turn on the instrument, and open the 7500 System software.

5.8.6.4. Create A Plate Document

5.8.6.4.1. On the Biomek computer, open the Biomek NX Workbook containing the set of samples being run, and click on the "RT1 Export" tab. If you have made any recent changes, save them now.

5.8.6.4.2. Select Save As→Other Formats. Open the Robot Team folder and select Save as type: "Text (Tab delimited)". Save the file.

5.8.6.4.3. Two alert windows will appear in Excel; click "OK" for the first and "Yes" for the second. Close the Biomek NX Workbook without making any further changes or saving again.

5.8.6.4.4. On the computer connected to the real-time PCR instrument, create a new plate document as described in the DNA Methods Manual.

5.8.6.4.5. Select File→Import Plate Setup. Import the text file you created in the Robot Team folder. The wells should populate with the names of the samples and standards.

5.8.6.4.6. Save the plate document and proceed with real-time PCR analysis as described in section 2 of the Forensic BiologySection Casework Test Method.

5.8.7. PowerPlex® 16 Hot-Start Amplification Prepared on the Biomek NX^{P} Laboratory Automation Workstation

5.8.7.1. Vortexing Extracts

5.8.7.1.1. Prior to amplification, the extract plate (the elution plate from the DNA IQ procedure) shall be vortexed on the Orbital Shaker module to ensure uniform pipetting of sample.

5.8.7.1.2. Spin down the extract plate to remove any condensation from the temporary seal.

5.8.7.1.3. In the Biomek Software, select "Instrument Menu" → "Manual Control."

5.8.7.1.4. Select the "Modules" menu and choose "Orbital Shaker."

5.8.7.1.5. Place the extract plate on the Orbital Shaker, with the temporary seal in place.

5.8.7.1.5.1. If the Orbital Shaker is clamped, change the command to "Unclamp" and click "Go."

5.8.7.1.6. Change the command to "Timed shake."

5.8.7.1.6.1. Enter 15 seconds for the time.

5.8.7.1.6.2. Enter 800 RPM for the speed.

5.8.7.1.6.3. Click "Go."

5.8.7.1.7. Change the command to "Unclamp." Click "Go."

5.8.7.1.8. Remove the extract plate from the Orbital Shaker.

5.8.7.1.9. Spin down the extract plate again before proceeding to amplification set-up. (CAUTION: Ensure that there are no bubbles in the wells.) Remove temporary seal.

5.8.7.2. Instrument Deck Setup

5.8.7.2.1. Open the "NormSTR_NXP_v1.0.0.ISP" method contained in the Promega project.

5.8.7.2.2. Place the following labware on the deck as shown in the figure below:

5.8.7.2.2.1. Box of P250 Span-8 Barrier Tips on the Rapid-Shuck module.

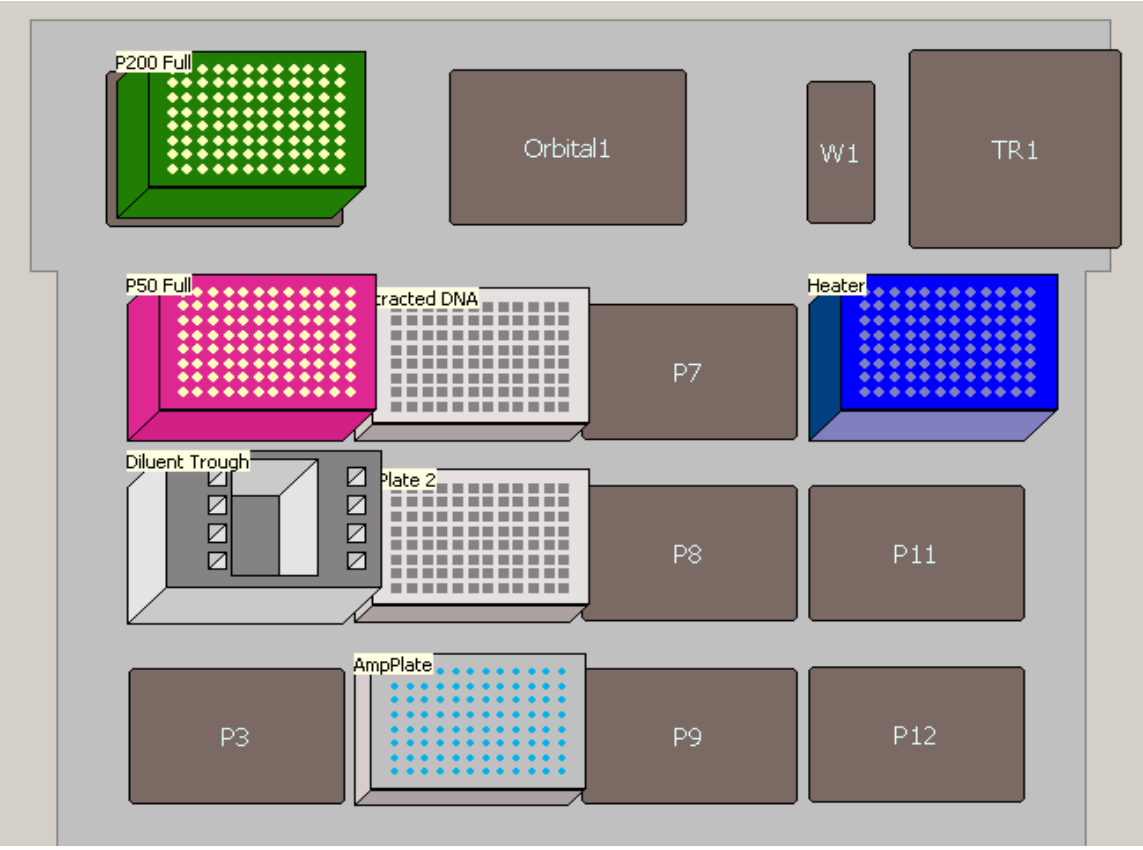
5.8.7.2.2.2. Box of P50 Barrier Tips on position P1. A second box of P50 Barrier Tips may be required on position P3.

5.8.7.2.2.3. 1.1 mL square-bottom deepwell plate on position P5. A second 1.1 mL square-bottom deepwell plate may be required on position P8.

5.8.7.2.2.4. New 96-well optical amplification plate on position P6.

5.8.7.2.2.5. Extract plate containing samples on position P4.

5.8.7.2.2.5.1. CAUTION: ensure that the MagnaBot module has been removed.



5.8.7.2.3. Click “Run.”

5.8.7.2.4. The program will prompt for three values:

5.8.7.2.4.1. First_P250_Tip = the number of the first unused tip in the 250µL tip box on the Rapid-Shuck module, numbering down columns and then across. Do not use a tip box with fewer than two columns of tips remaining. For a new box, enter 1.

5.8.7.2.4.1.1. *Example: If two and a half columns (twenty tips) of P250 tips have been used, enter “First_P250_Tip” as 21.*

5.8.7.2.4.2. First_P50_Tip = the number of the first unused tip in the 50 µl tip box on position P1, numbering down columns and then across. For a new box, enter 1.

5.8.7.2.4.3. Manual_MM_Prep =

5.8.7.2.4.3.1. Enter “True” (case sensitive) if you will prepare the master mix yourself by combining Primer Pair Mix and Reaction Mix from the PowerPlex®16 HS kit.

5.8.7.2.4.3.2. Enter “False” (case sensitive) if you would like the Biomek NX^{P} to prepare the master mix automatically.

5.8.7.2.4.3.2.1. NOTE: Ensure that there is sufficient Primer Pair Mix and Reaction Mix in each tube.

5.8.7.3. Normalization Manager

5.8.7.3.1. Login to the Normalization Manager with your username and password.

5.8.7.3.2. Enter a unique name for the amplification run, according to the batch number.

5.8.7.3.3. Select an analysis template according to the desired target input DNA to be amplified. Click “Next.”

5.8.7.3.4. Select the “Blanks_template” extraction control template. Click “Next.”

5.8.7.3.5. Select the “Default” injection control template. Click “Next.”

5.8.7.3.6. Browse for the appropriate .csv file exported by the 7500 System Software. Click “Open” and then “Next.”

5.8.7.3.7. Click “Review Extraction Plate” to check the samples that have been imported.

5.8.7.3.7.1. Wells that will **not** be amplified are colored gray or dark gray.

5.8.7.3.7.2. Any wells with a name containing “RB” should be orange (extraction control) and will be amplified unless otherwise specified.

5.8.7.3.7.3. Turn off amplification of appropriate samples by selecting the well and clicking “Amplify: No” at the lower left.

5.8.7.3.7.4. Turn off amplification of appropriate reagent blanks by clicking “Mark as Sample” at the top and then “Amplify: No” at the lower left.

5.8.7.3.7.5. When the appropriate samples have been selected for amplification, click “Done” and then “Next.”

5.8.7.3.8. Click “Review Amplification Plate” to check the re-arrangement of samples to be amplified.

5.8.7.3.8.1. Samples that will not be amplified have been removed.

5.8.7.3.8.2. Injection controls (AB, PC, and ladders) have been added.

5.8.7.3.8.3. The remaining samples to be amplified have been re-arranged. This is the final layout of both the amplification plate and the CE plate.

5.8.7.3.8.4. Delete extra ladders (such as at the end of a half-full plate) by right-clicking them.

5.8.7.3.8.4.1. CAUTION: Wells H2, A4, H6, A8, H10, and A12 must never contain samples. These wells will have ladders automatically added during the CE Setup step using the Biomek 3000.

5.8.7.3.8.5. When you are satisfied with the amplification plate, click “Done” and then “Next.”

5.8.7.3.9. Select the desired CE Instrument Injection Protocol from the drop-down list. Additional protocols, if desired, may be added in the CE Instrument Software.

5.8.7.3.10. Re-enter your username and password when requested, and click “Finish.”

5.8.7.3.11. The Normalization Manager will exit automatically and the protocol will continue in the Biomek Software.

5.8.7.4. Reagent Preparation

5.8.7.4.1. Follow the prompts within the Biomek Software. Note that depending on the data loaded in the Normalization Manager step, the protocol may require an additional 1.1 ml square-bottom deepwell plate on position P8.

5.8.7.4.2. If you have opted for the Biomek NX^{P} to prepare the STR Master Mix automatically, follow the prompts.

5.8.7.4.3. If you are making your STR Master Mix manually, a prompt will provide the required volumes for each component.

5.8.7.4.4. Place the STR Master Mix tube in position 4 (front-most) of the Left Tube Holder on the reagent rack.

5.8.7.4.5. Place the tube containing diluted positive control (at least 20 µl) in position 1 (rear-most) of the Right Tube Holder on the reagent rack. Click “OK.”

5.8.7.4.6. Pipet the indicated amount of amplification-grade water or nuclease-free water into the center trough of the reagent rack. Place the reagent rack on deck position P2.

5.8.7.5. Running the Normalization/Amp Set-up Method

5.8.7.5.1. Check that the deck layout matches the layout indicated in the next prompt. Click "OK."

5.8.7.5.2. The run will begin, performing normalization and amplification setup in one step.

5.8.7.5.3. When the run is finished, remove the amplification plate and cover with adhesive foil. Press the foil well using a roller tool.

5.8.7.5.4. Remove the elution plate containing extracted samples and cover with a temporary seal and refrigerate.

5.8.7.6. Create and Export Amp/CE Plate Record

5.8.7.6.1. Open the "*platename*_CEReport" text document created by the Normalization Manager, contained in the folder "AnalysisRunPerformed." Select All and copy.

5.8.7.6.2. Open the Biomek Workbook containing the set of samples that have been run, and click on the "Amp Plate Import 1" tab. Click on the red cell in the upper-left corner, and paste.

5.8.7.6.3. Click on the "Amp Plate View" tab. The sample names and locations have been imported to the plate worksheet, and the plate name and operator have been filled in.

5.8.7.6.4. Click on the "Insert Cases" button. The case numbers for all samples on the plate are compiled in the "Cases" box.

5.8.7.6.5. Fill out the rest of the worksheet as designated, including dates, reagent lot numbers, amplification reaction type, and injection protocols.

5.8.7.6.6. Save the completed worksheet as a .pdf file.

5.8.8. Preparing a 3130 plate on the Biomek 3000

5.8.8.1. Biomek 3000 Preparation

5.8.8.1.1. Open the "Biomek Software" located on the desktop.

5.8.8.1.2. Calibrate the Biomek 3000 prior to operation.

5.8.8.1.3. All tubes of allelic ladder in the post-amplification kit shall be combined into a single 1.5 ml Eppendorf microcentrifuge tube (do not use a "dolphin"-style tube), marked as "ladder" with associated lot numbers recorded on the tube, and stored with the kit when not in use.

5.8.8.2. Instrument Deck Setup

5.8.8.2.1. Click File → Open, select “Direct_transfer_with_ladder”.

5.8.8.2.1.1. This will open the designated program that will transfer a plate with dedicated ladder positions into the A4, A8, A12, H2, H6, and H10 positions.

5.8.8.2.2. Place the following labware on the deck as shown in the figure below:

5.8.8.2.2.1. Box of Beckman Coulter Biomek® AP96 P250 Tips (Cat. no. 717253) on the ML1 position.

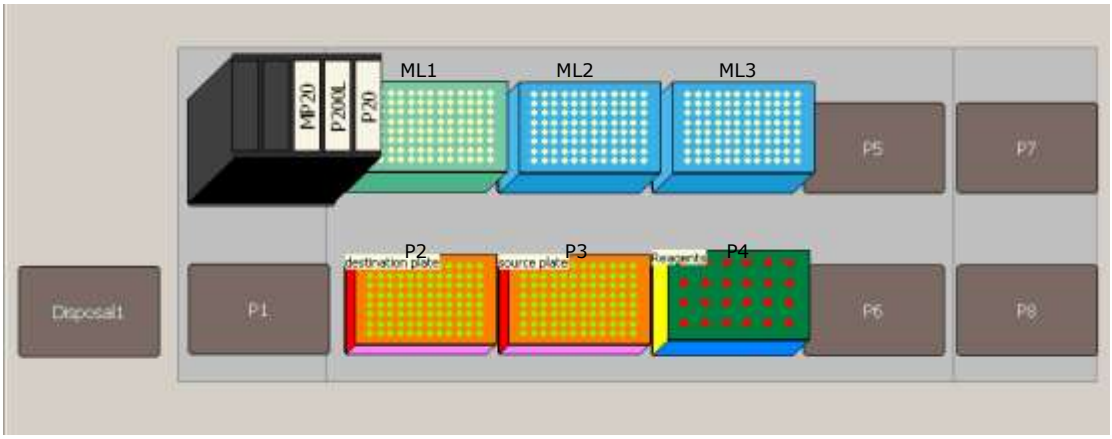
5.8.8.2.2.2. Two boxes of Beckman Coulter Biomek® AP96 P20 Tips (Cat. no. 717256) on the ML2 and ML3 positions.

5.8.8.2.2.2.1. Note: A typical plate setup will utilize one P250 tip, eight P20 tips per column transferred, and between one and six P20 tips for ladder transfers. Ensure that enough tips are available before proceeding.

5.8.8.2.2.3. One new 96-well optical plate at position P2.

5.8.8.2.2.3.1. This is the CE plate where master mix and samples will be loaded when the program has finished.

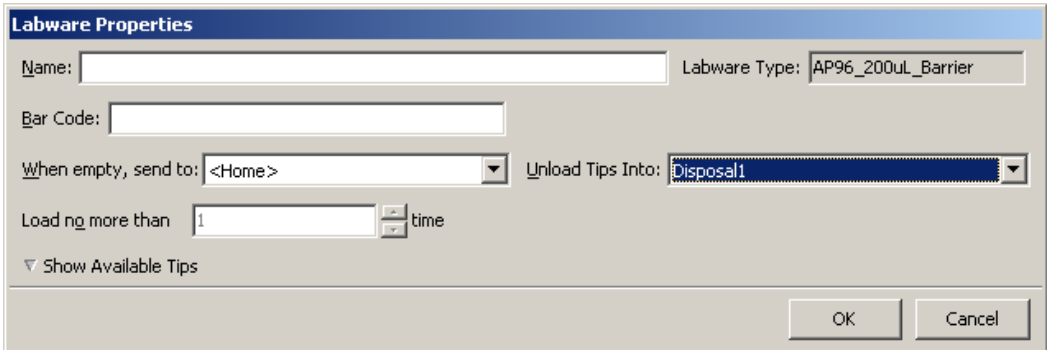
5.8.8.2.2.4. Spin down the amplification plate and ensure that no bubbles remain. Place the plate in deck position P3. Secure the amplified plate to the plate rack with tape.



5.8.8.2.3. In the software program, click the “Instrument Setup” step, which will bring up the Deck Display.

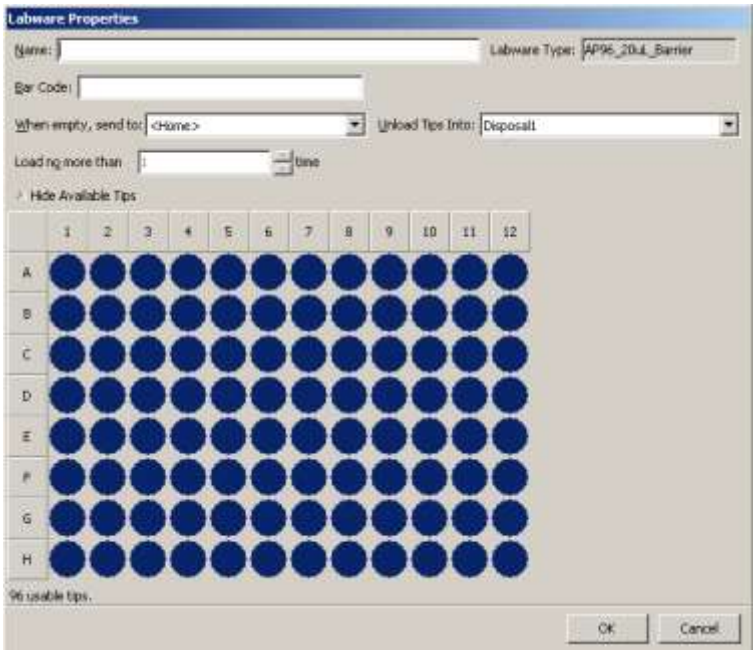
5.8.8.2.4. Right-click on P250 box in position ML1 and select “Properties” to open the “Labware Properties” menu as shown below.

5.8.8.2.5. Ensure that the “Unload Tips Into:” drop down menu is selected as “Disposal1”, the “When empty, send to:” drop down menu is selected as “<Home>”, and the “Load no more than” drop down menu is designated as “1”.



5.8.8.2.6. Select “Show Available Tips” as shown below.

5.8.8.2.7. By clicking on individual tip locations, you may designate whether tips are present (blue) or absent (white). For every location where a tip is absent from the physical deck, click the appropriate location to turn that location white. You may reference the bottom left corner of the menu to determine the number of available tips that are being designated as usable.



5.8.8.2.8. Configure Labware Properties and available tips for the P20 boxes located at positions ML2 and ML3 in the Deck Layout.

5.8.8.3. Reagent Preparation

5.8.8.3.1. To prepare master mix for automated dispensing by the Biomek 3000:

5.8.8.3.2. In a 1.5 ml Eppendorf microcentrifuge tube, prepare the appropriate volume of master mix (Hi-Di and ILS) for the associated number of samples and/or columns (see example chart below for 1.0 µl ILS/sample).

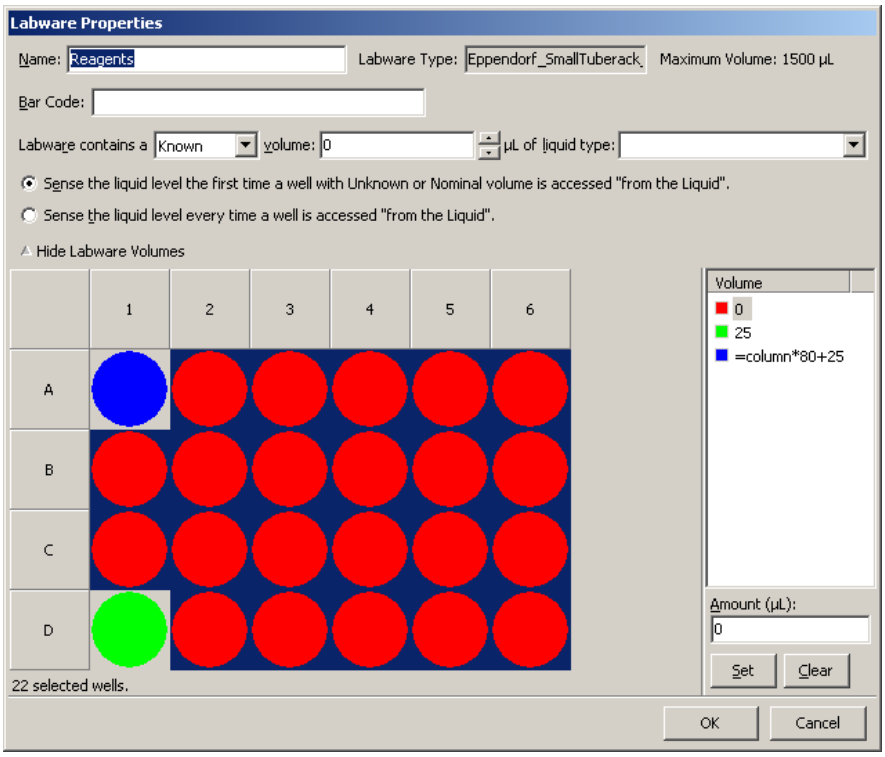
Number of Samples+Ladders	Number of Columns	ILS (ul) (1.0 ul/sample)	Hi-Di (ul)	Total (ul)
1-16	2	18	162	180
17-32	4	34	306	340
33-48	6	50	450	500
49-64	8	66	594	660
65-80	10	82	738	820
81-96	12	98	882	980

5.8.8.3.2.1. Do not use a “dolphin”-style microcentrifuge tube, or the robot may fail to load master mix accurately to the columns.

5.8.8.3.2.2. Ensure that sufficient master mix is prepared to cover all capillaries during electrophoresis, or manually pipet Hi-Di into empty wells after the plate is complete.

5.8.8.3.3. Vortex and spin down, ensuring that no bubbles remain.

5.8.8.3.4. Place the prepared master mix tube in position A1 of the 24-well tube rack located in position P4 on the Deck, as shown in blue below.



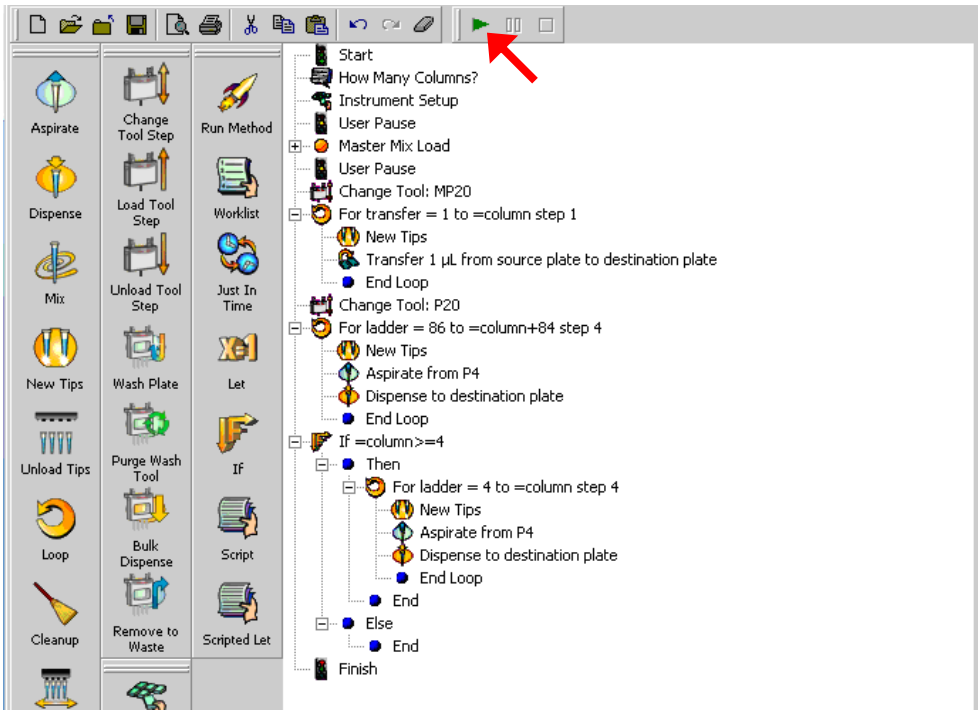
5.8.8.3.5. Vortex and quickly spin down the combined tube of allelic ladder and ensure that no bubbles remain.

5.8.8.3.6. Place the tube of ladder in position D1 of the 24-well tube rack located in position P4 on the Deck, as shown in green above.

5.8.8.4. Starting the CE Plate Set-up Run

5.8.8.4.1. Remove all lids from tip boxes and from the tubes of prepared master mix and ladder and place them clear of the deck.

5.8.8.4.2. In the software program, click on the green “run” arrow located on the top command bar, as shown below.



5.8.8.4.3. Follow the prompts as they appear. You will be asked to input the number of columns for the plate setup. This number should be the same as originally determined when preparing the master mix (see chart above).

5.8.8.4.4. When the program has completed, remove and discard any empty tip boxes.

5.8.8.4.5. Discard the master mix tube, and store the allelic ladder tube with the post-amplification kit.

5.8.8.4.6. Seal the amplification plate with aluminum foil plate seal and store frozen.

5.8.8.4.7. Add a septa cover to the CE plate, and load onto genetic analyzer as described in section 2 of the Forensic BiologySection Casework Test Method.

5.8.9. Final Tube Transfer

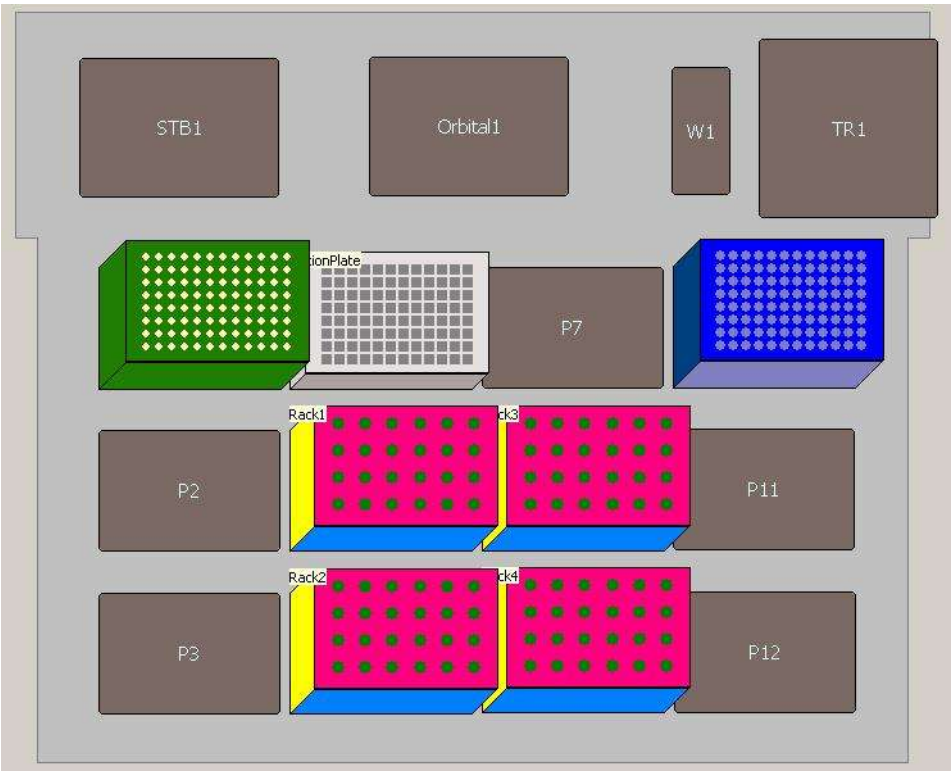
5.8.9.1. Place new, empty, labeled Molecular BioProducts (San Diego, CA) 1.5 ml screw-top tubes (Cat. no. 3474) in the tube racks (4x6 with white adapters), arranging the tubes according to the order shown in the Biomek Workbook. Do not skip columns or spaces between samples.

5.8.9.2. Arrange the deck layout as shown in the figure below:

5.8.9.2.1. Place sample tube racks on positions P5, P6, P8, and P9, again paying attention to sample order according to the Biomek Workbook.

5.8.9.2.2. Place the plate containing extracted samples on position P4.

5.8.9.2.3. Place a new box of Beckman Coulter Biomek® P250 Span-8 Barrier Tips on position P1.



5.8.9.3. Open the "Final Plate to Tube Transfer" method contained in the Promega project.

5.8.9.4. Click the green "Run" button at the top of the screen.

5.8.9.4.1. The method will prompt for a value equaling the number of full or partial columns of sample on the extraction plate.

5.8.9.4.1.1. *Example: 20 samples/blanks will use two full columns and half of a third. Enter "SampleColumns" as 3.*

5.8.9.5. When the instrument has finished transferring sample from the extraction plate to the tubes, remove the tube racks from the deck, cap the screw-top tubes, and discard the empty sample plate.

5.8.10. When processing is completed, the submitting analyst will be notified by e-mail that the data is available on the DNA server and the extracted DNA samples can be retrieved from a designated location.

5.9. Records

5.9.1. N/A

5.10. Interpretation of Results

5.10.1. N/A

5.11. Report Writing

5.11.1. N/A

5.12. References

5.12.1. N/A

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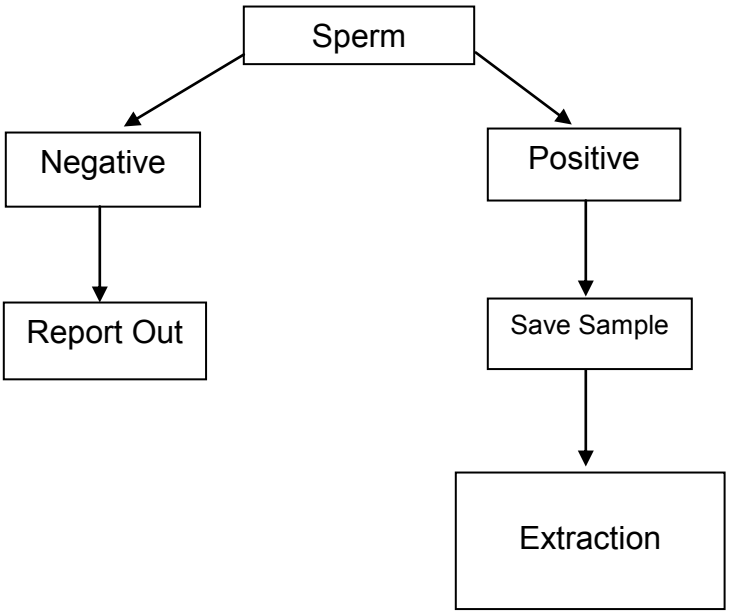
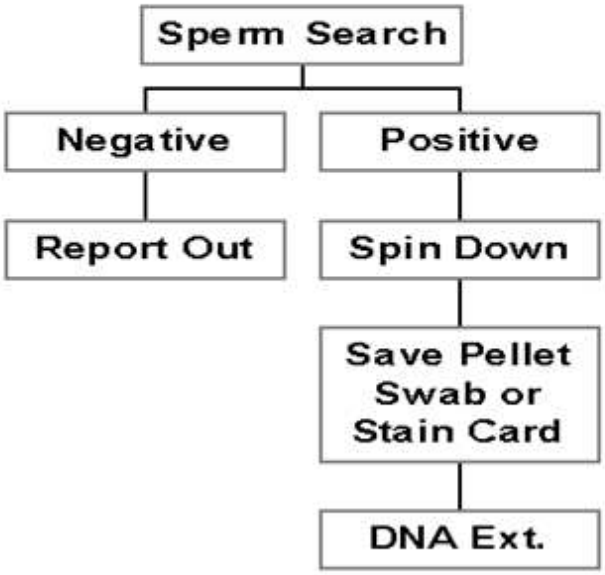
TEST METHODS
APPENDIX 1 SEROLOGY FLOW CHARTS

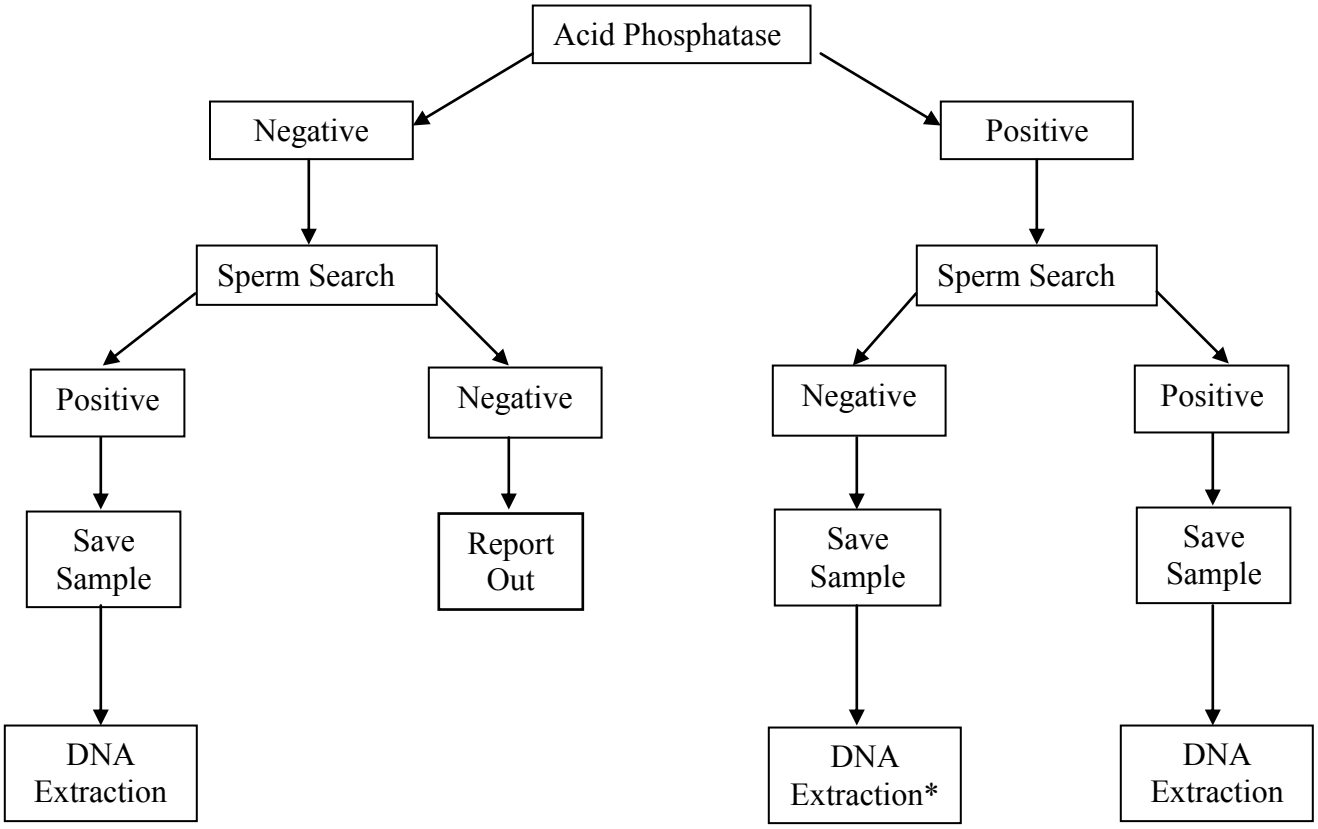
Smear Slides
Semen Identification



Vaginal Wash
Semen Identification



INDIANA STATE POLICE
FORENSIC BIOLOGY SECTION
TEST METHODS
Swabs for Semen Identification



* Optional when other samples extracted.

Amylase testing may be performed at analyst's discretion.

Dental floss, swabs indicated as bitemark or dried secretions swabs, and any swabs collected from male genitalia may be sent directly to DNA analysis. Other exceptions shall have Supervisor's Approval.

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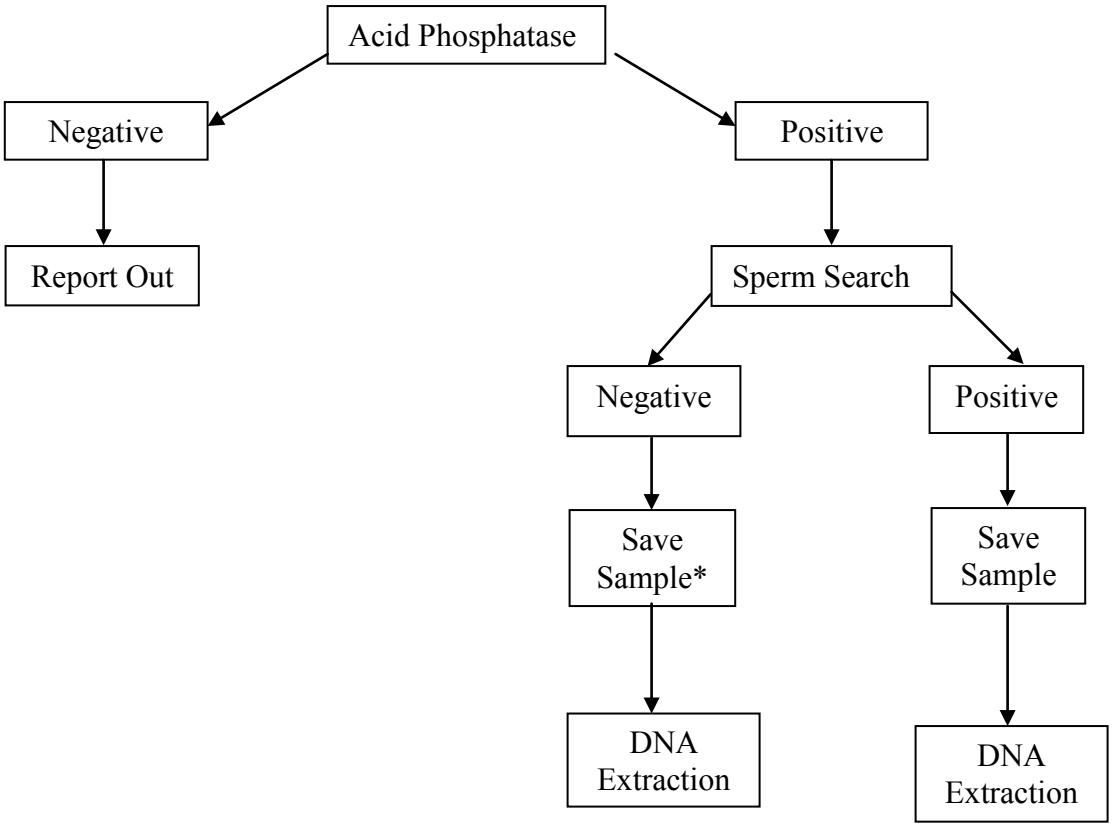
TEST METHODS
Other Items (e.g. clothing, bedding, etc.) for Semen Identification



*If the Acid Phosphatase test is the only positive result, the sample should be sent for DNA analysis.

Amylase testing may be performed at analyst's discretion.

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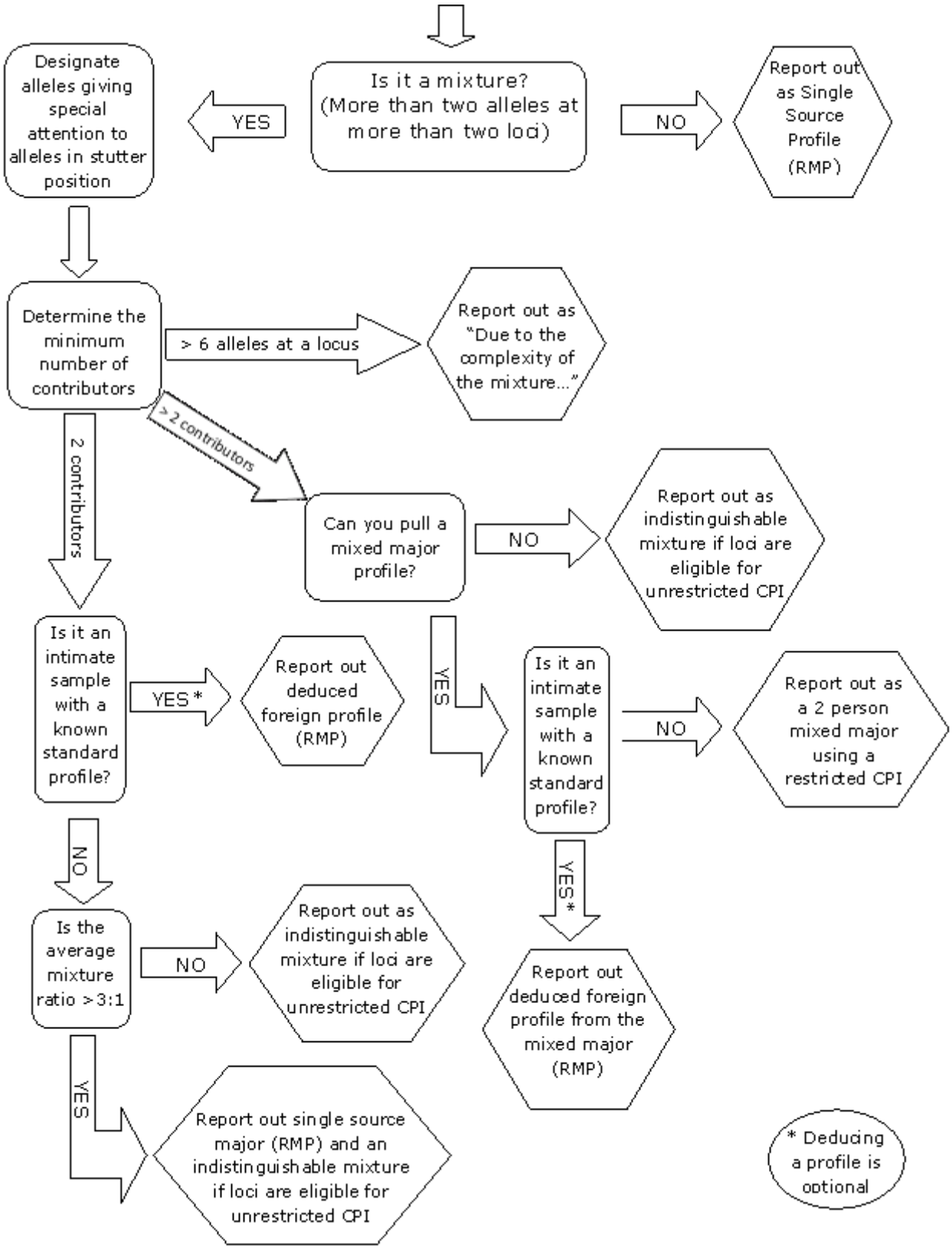
FORENSIC BIOLOGY SECTION
TEST METHODS
APPENDIX 2
MIXTURE INTERPRETATION FLOW CHART


Is it a mixture? (More than two alleles at more than two loci)
YES
Designate alleles giving special attention to alleles in stutter position
NO
Report out as Single Source Profile (RMP)
Determine the minimum number of contributors
> 6 alleles at a locus
Report out as "Due to the complexity of the mixture..."
> 2 contributors
2 contributors
Can you pull a mixed major profile?
NO
Report out as indistinguishable mixture if loci are eligible for unrestricted CPI
Is it an intimate sample with a known standard profile?
YES *
Report out deduced foreign profile (RMP)
YES
Is it an intimate sample with a known standard profile?
NO
Report out as a 2 person mixed major using a restricted CPI
NO
YES *
Is the average mixture ratio >3:1
NO
Report out as indistinguishable mixture if loci are eligible for unrestricted CPI
Report out deduced foreign profile from the mixed major (RMP)
YES
Report out single source major (RMP) and an indistinguishable mixture if loci are eligible for unrestricted CPI
* Deducing a profile is optional

APPENDIX 3 DEFINITIONS

1. Acid Phosphatase – a chemical component of semen; also found in other body fluids at significantly lower concentrations.
2. Allele – the alternative form of a gene.
3. Allele Frequency – the proportion of a particular allele found in a population.
4. Allelic Ladder – a set of DNA fragments of the commonly known alleles for each locus. By comparing the samples to the allelic ladder, the correct allele designation may be assigned.
5. Amelogenin – the marker for determining the gender of the individual contributor to a DNA profile.
6. Amplification – using the PCR process to create many copies of a specific DNA sequence(s). An increase in the number of copies of a specific DNA fragment.
7. Amylase – a chemical component of saliva; also found in other body fluids.
8. Analyst Discretion - The use of individual judgment, based on an analyst's training and experience to determine the optimum modes of analysis for an item of evidence.
9. Artifact – non-allelic product of the amplification process, an anomaly of the detection process, or a by-product of primer synthesis. A data peak that does not represent a true allele.
10. Base Pairs – Paired nucleotides which make up the DNA molecule. Two complementary nucleotides joined by a hydrogen bonds.
11. Blood – the fluid that circulates through the body, containing red and white blood cells carrying oxygen and nutrients to cells throughout the body; and carrying away waste and carbon dioxide.
12. Capillary Electrophoresis – a method to separate DNA fragments based on size using electrical current. The DNA sample is placed in thin tube (capillary) containing gel (polymer) and subjected to high voltage current allowing the DNA fragments to migrate through the tube.
13. Chromosome – the structure on which genes are naturally arranged and how DNA is organized.
14. Combined DNA Index System (CODIS) – refers to the DNA database and its software. It is composed of National (NDIS), State (SDIS), and Local (LDIS) components. It contains DNA profiles from offenders, crime scenes and includes a missing person database.
15. Combined Probability of Inclusion/ Combined Probability of Exclusion (CPI/CPE) – statistical method for calculating the inclusion/exclusion of a random person in a profile that has more than one person present.
16. Contamination – the process of making a sample impure or unusable.
17. Deduced – the inference of an unknown contributor's DNA profile after taking into consideration the contribution of an assumed contributor's DNA profile in an intimate sample based on quantitative peak height information.
18. Degradation – the breaking down of the DNA molecule into smaller fragments.
19. Denaturation – the separation of the double stranded DNA molecule into two single strands.
20. Deoxyribonucleic Acid (DNA) - the genetic material present in the nucleus of most cells. Also located in cell mitochondria.
21. DNA Sequence - a specific order of base pairs.
22. Electropherogram – the visual representation of the DNA fragments contained in each sample; generated by the analysis software of the Capillary Electrophoresis Instrument.
23. Electrophoresis – the method to separate molecules based on their size by placing them in a medium and applying a electrical current. The molecules will travel through the medium at different rates, the smaller molecules traveling through the medium more quickly than the larger ones.

24. Enzyme – a protein which acts as a catalyst, speeding up a specific chemical reaction without being changed or consumed in the process.
25. Epithelial Cells – skin cells and other surface cells such as from the mouth (buccal cells) or the vaginal cavity.
26. Gene – the basic functional unit of heredity. Most genes determine the structure and function of proteins.
27. Genotype – the specific genetic sequence of a person's DNA.
28. Hair – a slender outgrowth from the skin of mammals.
29. Heme – the iron containing complex of the hemoglobin molecule.
30. Hemin – the oxidized heme molecule.
31. Hemoglobin - the iron containing protein in blood.
32. Heterozygous – having different alleles at a particular locus.
33. Homozygous – having two of the same allele at a particular locus.
34. Internal Lane Standard (ILS) – a set of DNA fragments of known length(s). The ILS is simultaneously injected with all DNA samples during electrophoresis. This allows accurate measurement of the length of each allele in a DNA sample.
35. Intimate samples – Biological sample that is obtained directly from an individual's body.
36. Locus (loci) – the physical location of a gene on a chromosome.
37. Marker – a gene of known location and phenotype used as a point of reference.
38. Meaningful profile – A profile that is developed from an item or location where the individual's profile would not reasonably be expected. A person's profile may be expected on an item or sample from their own body, their own clothing, or their property such as their house or vehicle. All other samples will generally be considered meaningful.
39. Mitochondria - a part of a cell used in energy production. Contains circular DNA inherited from the mother only.
40. Nucleus - the part of a cell that contains the double stranded DNA inherited from both parents.
41. Obligate allele – an allele in a mixed DNA typing result that is a) foreign to an assumed contributor, or b) based on quantitative peak height information, determined to be shared with the assumed contributor.
42. Partial profile – DNA profile for which typing results were not obtained for all tested loci.
43. Phenotype – the expressed genotype.
44. Polymerase – an enzyme that initiates the duplication of a DNA molecule.
45. Polymerase Chain Reaction (PCR) – a process for amplifying (copying) the DNA molecule.
46. Polymorphism – the presence of more than one possible allele set for a specific gene.
47. Population – a stable group of random individuals, chosen for Genetic analysis.
48. Primer – a short nucleotide fragment of known sequence used to locate its complementary sequence on the DNA molecule for the initiation of PCR. Primers target the specific loci to be amplified.
49. Proficiency Testing – a test to evaluate the competency of an analyst in a specific procedure.
50. Restricted Combined Probability of Inclusion (CPI) – a statistical approach utilizing consideration of quantitative peak height information and inference of contributor mixture ratios to limit the genotypic combinations of possible contributors included in the CPI calculation.
51. Random Match Probability (RMP) - the probability of obtaining a match between two distinct and unrelated individuals.
52. Saliva – digestive fluid from the mouth. Saliva contains amylase.
53. Semen/Seminal Fluid – the male ejaculate. In a dried state may be referred to as seminal material. Semen normally contains spermatozoa.

54. Short Tandem Repeat (STR) – small sections of DNA that contain short segments (2, 3, 4 or more base pairs) which repeat several times. The number of repeat units may vary between individuals. STRs are located between specific genes and are considered non-functional.
55. Spermatozoa – the male reproductive cell, sperm cell.
56. Stochastic effect – peak imbalance observed in a locus and/or allele drop-out due to random, disproportionate amplification of alleles in low quality/quantity template samples.
57. Stutter – an artifact that occurs as a by-product of the PCR process. It is observed as a minor peak typically observed one repeat unit smaller than a primary STR allele caused by strand slippage during amplification.
58. Substrate – the material on which a biological sample is deposited such as at a crime scene.
59. SWGDAM – Scientific Working Group on DNA Analysis Methods.
60. Unrestricted Combined Probability of Inclusion (CPI) – a statistical approach when the CPI is performed without consideration of quantitative peak height information and inference of contributor mixture ratios.
61. Validation – a study to assess whether a particular procedure (or instrument) can obtain a desired result reliably and reproducibly. The study includes looking at the conditions necessary to obtain those results and the limitations of the procedure/instrument. All DNA analysis methods shall be validated prior to implementation.
62. Work Product – the material that is generated as a function of analysis, which may include extracts and amplified product, in tubes or plates, and any aliquots thereof.
63. X Chromosome – a sex chromosome, present twice in female cells and once in male cells.
64. Y Chromosome – a sex chromosome, present once in male cells (and absent in female cells), paired with the X chromosome.

APPENDIX 4 STATISTICS REFERENCE SHEET

STR population stats:

Budowle B., Moretti T.R., Baumstark A.L., Defenbaugh D.A., Keys K.M. Population data on the thirteen CODIS core short tandem repeat loci in African Americans, U.S. Caucasians, Hispanics, Bahamians, Jamaicans, and Trinidadians. J. Forensic Sci. 1999; 44(6): 1277-1286.

Smith, J., Budowle, B. Source Identification of Body Fluid Stains Using DNA Profiling. Proceedings of the Second European Symposium, Innsbruck, Austria. 6/98

Budowle, B., Chakraborty, R., Carmody, G., Monson,K., Source Attribution of a Forensic DNA Profile. *Forensic Science Communications.* July 2000, Volume 24, Number 3.

Budowle, B., Shea, B., Niezgoda, S., Chakraborty, R. CODIS STR Loci Data from 41 Sample Populations. J. Forensic Sci. 2001; 48(3): 453-489

Levandakou, E. N., et al. Allele Frequencies for Fourteen STR Loci of the Powerplex™ 1.1 and 2.1 Multiplex Systems and Penta D Locus in Caucasians, African-Americans, Hispanics, and Other Populations of the United States of America and Brazil. J. Forensic Sci. 2001; 48(3): 736-761.

Null allele frequency: Pmin = M/n
Where M = 5 and n = the total number of alleles in data file.

Homozygous alleles are calculated using the formula:
allele frequency^2+ allele frequency(1-allele frequency)θ. Where θ is 0.01.

Heterozygous alleles are calculated using the formula:
2pq. Where p = frequency of 1st allele and q = frequency of 2nd allele.

Mixtures

P = (pA+pB+ . . .)
Q = 1-p
PE = q^2 +q(θ)(1-q) + 2q(1-q)(1- θ)
PI = p^2 +p(1-p)(θ)
CPE = 1-(1-PE_{D16})(1-PE_{D7})(…)
CPI = (PI_{D16})(PI_{D7})(…)

Source Attribution

$p \leq 1-(1-\alpha)^{1/N}$
Where p equals adjusted probability
α equals 0.01, representing a confidence level of 99%
N equals 330,000,000; a conservative population size value equal to the population of the United States.

APPENDIX 5 DNA SERVER INSTRUCTIONS

The DNA servers will be used in place of CDs for storage and archiving of data and photos. Real Time PCR SDS files, 3130 run folders, GeneMapper® *ID-X* projects and photos will be saved to the server. Any worksheets, statistic sheets, typed notes pages, or workbooks shall NOT be saved to the server. The printed copy that will be scanned into LIMS upon completion of the case will be the official, tracked copy. We want to avoid duplication of items between LIMS and the server.

On the server, each analyst will have a folder that only he will be able to write to. Other analysts will be able to view items in the folder, but will not be able to change, delete or add items within the folder. Within the analyst's folder there will be one folder for each lab file number and request. Each folder will contain all associated data for that lab file request. Any projects or run folders that contain batched data will be saved individually within each of the associated lab file folders. The following nomenclature shall be used:
Analyst's Folder (created by Lab IT)

- Lab file and request folder – "labfile_request number" – ex. "10I1234_1" – the request number corresponds to the laboratory request number assigned in LIMS.
    - RT-PCR SDS projects: "1st labfile on the plate_date_plate#" – ex. "10I1234_01Jan11_01"
    - 3130 run folder: "1st labfile on the plate_date_plateinjection#" – ex. "10I1234_01Jan11_01" – plate injection number indicates how many times that plate preparation has been put in the instrument for injection; if the same plate preparation is run on a different date, the original date should still be used from when the plate was prepared. If a second preparation of a plate is made on the same date as the first, it shall be designated with a "-2" after the date. Ex. "10I1234_01Jan11-2_01"
    - GeneMapper® *ID-X* project name: must contain the injection protocol and date the run was started (in most cases the same as the date in the run folder name)- ex. "3kV3sec_01Jan11", "10I1234_3kV3sec_01Jan11" or "Controls_3kV3sec_01Jan11"
    - Photos: create a folder per item photographed – folder name "lab file_item#" ex. "10I1234_item1". The individual photo file names are left to the analyst's discretion.

Note: RT-PCR and 3130 Plates generated by the automation team may substitute Batch ID # for Lab File #.

Note: For RT-PCR SDS projects and 3130 run folders – only the first lab file on the plate should be included in the name. Do not write more than one lab file number and do not write "et al." after the name.

APPENDIX 6 RELATIONSHIP COMPARISON STATISTICAL REFERENCE SHEET

Formulas for Paternity Index (PI) and Random Man Not Excluded (RMNE) *(Table below adapted from the American Association of Blood Banks, Standards for Relationship Testing Laboratories, 9th Edition, Appendix 7 and 8)*

Mother	Child	Alleged Father	PI	RMNE	PE
BD	AB	AC	1/2a	$1-(1-a)^2$	$(1-a)^2$
BC	AB	AC	1/2a	$1-(1-a)^2$	$(1-a)^2$
BC	AB	AB	1/2a	$1-(1-a)^2$	$(1-a)^2$
BC	AB	A	1/a	$1-(1-a)^2$	$(1-a)^2$
B	AB	AC	1/2a	$1-(1-a)^2$	$(1-a)^2$
B	AB	AB	1/2a	$1-(1-a)^2$	$(1-a)^2$
B	AB	A	1/a	$1-(1-a)^2$	$(1-a)^2$
AB	AB	AC	1/[2(a+b)]	$1-(1-a-b)^2$	$(1-a-b)^2$
AB	AB	AB	1/(a+b)	$1-(1-a-b)^2$	$(1-a-b)^2$
AB	AB	A	1/(a+b)	$1-(1-a-b)^2$	$(1-a-b)^2$
AB	A	AC	1/2a	$1-(1-a)^2$	$(1-a)^2$
AB	A	AB	1/2a	$1-(1-a)^2$	$(1-a)^2$
AB	A	A	1/a	$1-(1-a)^2$	$(1-a)^2$
A	A	AB	1/2a	$1-(1-a)^2$	$(1-a)^2$
A	A	A	1/a	$1-(1-a)^2$	$(1-a)^2$
	AB	AC	1/4a	$1-(1-a-b)^2$	$(1-a-b)^2$
	AB	AB	(a+b)/4ab	$1-(1-a-b)^2$	$(1-a-b)^2$
	AB	A	1/2a	$1-(1-a-b)^2$	$(1-a-b)^2$
	A	AC	1/2a	$1-(1-a)^2$	$(1-a)^2$
	A	A	1/a	$1-(1-a)^2$	$(1-a)^2$

Paternity Index for Mutations

PI = μ/ [PE] μ = mutation rate for the locus

Combined Paternity Index (CPI)

$CPI = PI_1 \times PI_2 \times PI_3 \ldots..$

Probability of Paternity (W)

W = CPI x Pr / [CPI x Pr + (1-Pr)]

Formulas for Reverse Paternity

(Table below adapted from the American Association of Blood Banks, Guidelines for Mass Fatality DNA Identification Operations, 2010, Appendix B)

Mother	Possible Child	Father	Formula
A	A	AB	$1/2a^2$
A	AB	AB	1/4ab
A	AB	BC	1/4ab
AB	A	AB	$1/4a^2$
AB	A	AC	$1/4a^2$
BC	AB	AB	1/8ab
BC	AB	AC	1/8ab
BD	AB	AC	1/8ab
A	A	A	$1/a^2$
AB	A	A	$1/2a^2$
B	AB	A	1/2ab
BC	AB	A	1/4ab
AB	AB	AC	1/8ab
AB	AB	A	1/4ab
AB	AB	AB	1/4ab

Formulas for Sibship

(Table below adapted from the American Association of Blood Banks, Guidelines for Mass Fatality DNA Identification Operations, 2010, Appendix B)

Sibling	Possible Full Sibling	Formula
AB	AB	(1+a+b+2ab)/8ab
A	A	$(1+a)^2/(2a)^2$
A	AB	(1+a)/4a
AB	AC	(1+2a)/8a
AB	CD	1/4

Half Sibling	Possible Half Sibling	Formula
AB	AB	(a+b+4ab)/8ab
A	A	(1+a)/2a
A	AB	(1+2a)/4a
AB	AC	(1+4a)/8a
AB	CD	1/2

STR Population Data

Budowle, B., Shea, B., Niezgoda, S., Chakraborty, R. CODIS STR Loci Data from 41 Sample Populations. J. Forensic Sci. 2001; 48(3): 453-489

Levandakou, E. N., et al. Allele Frequencies for Fourteen STR Loci of the Powerplex™ 1.1 and 2.1 Multiplex Systems and Penta D Locus in Caucasians, African-Americans, Hispanics, and Other Populations of the United States of America and Brazil. J. Forensic Sci. 2001; 48(3): 736-761.